The understood of nutrients and energy transfers in aquatic ecosystems has long been a major focus of ecological research. Studies of nitrogen cycling are a cornerstone of ecosystem biogeochemistry because all biota depend on N for critical processes, being recognized as key element in aquatic ecosystems and controlling global productivity. This dissertation provides insights into global inland water ecosystems functioning: what controls community structures of lakes, N biogeochemical transformations, and the role of different N pools in rivers and lakes. This thesis also has great implications for the use of stable isotopes (^{15}N and ^{13}C), natural abundances and deliberate additions, in limnological studies.

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TESIS DOCTORAL

The use of stable isotopes to assess food web structure and nitrogen dynamics in freshwaters

Celia Ruiz Jiménez

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A mi madre y a mi padre,
por quererme tanto

"Todos somos científicos locos, y la vida es el laboratorio, estamos tratando de experimentar para encontrar una manera de vivir, para resolver problemas, para defenderse de la locura y el caos"

David Cronenberg

CONTENTS

A. GENERAL INTRODUCTION

INTRODUCCIÓN GENERAL

"One cannot manage aquatic ecosystems effectively without understanding how they operate in response to interaction of physical, chemical, and biotic environmental variables"

Robert G. Wetzel

Inland water ecosystems (mainly lakes, reservoirs and rivers) are only a tiny part of the earth´s surface, but their importance in terms of ecological meaning and human services is well recognized (Lamper and Sommer, 1997; Wetzel, 2001; Carpenter et al., 2011; Cosgrove et al., 2014). During the last century, these ecosystems have been dramatically impacted by global and local scale changes, experiencing accelerating rates of degradation (e.g. eutrophication, loss of biodiversity and pollution; Wetzel, 2002; Shurin et al., 2012). One of the major pressures on these aquatic ecosystems is that associated to the alteration of the nitrogen (N) cycle, which may have already exceeded biophysical thresholds of recovery (Rockström et al., 2009). Given the negative consequences of these impacts, a complete understanding of inland water ecology is becoming increasingly important to confront and to predict the present and future health of freshwater resources (Wetzel, 2001; Saunders and Kalff, 2001; Dodds, 2002, 2013; Mantyka-Pringle et al., 2014).

To improve the knowledge of the mechanisms and dynamics underlying the ecology of aquatic ecosystem, limnologists reduce the holistic view of ecosystem to manageable units, making simplifications and developing generalizations (Jopp et al., 2011). Approaches related to ecosystem structure and nutrient dynamics have been extensively used to characterize communities and networks among species, to define biotic compartments, links with environmental attributes and functional processes (Pimm, 2002; Dodds, 2002; Mutshinda and O'Hara, 2011; Cardinale, 2011; Thompson et al., 2012; Abrantes et al., 2014). Both characterizations are key features of ecosystems and have been defined as fundamental tools in the study of aquatic ecology (Wetzel, 2002; Glibert et al., 2011; Sardans et al., 2011).

A.1 FOOD WEB STRUCTURE

Ecosystems have a structure, in the sense that they are composed of different elements, biotic and abiotic, and these are arranged creating a network of interactions following definite patterns (Margalef, 1963; Keller and Golley, 2000). During decades, different measurements have been developed to assess numerous aspects of the structure attributes, as species richness (e.g. Shannon–Wiener Index; Shannon and Weaver, 1948), biotic integrity (e.g. Integrity Biotic Index IBI; Karr, 1981) and resources features (e.g. Allochthonous *vs.* autochthonous inputs; Sterner et al., 1997). Among the most important interactions within an ecosystem are those of trophic nature. Then, feeding relationships and food webs have been extensively used to assess and characterize ecosystem structure (Lindemans, 1942; Scheffer, 1998; Vander Zanden et al., 2003; Abrantes et al., 2014; Middelburg, 2014).

Unraveling the complexities of food web structure and dynamics can be exceedingly difficult (Winemiller and Layman, 2005). Tools used to this end include the assessment of trophic diversity, ecological niche and species packing (Layman et al., 2007; Newsome et al., 2007; Schmidt et al., 2007). Descriptors based on the total number of species, as number of trophic links, food chain length, evenness and connectance are also of central importance in theories of community structure (May, 1986; Lawton, 1989; Warren, 1990). Each of these approaches has distinct strengths and weaknesses, focusing on specific characteristics of food webs. The quantification of as many aspects as possible, results in a better approximation of the real food-web interactions and by hence, of ecosystem structure.

All types of freshwaters have food web structures that are similar in some aspects (e.g. connectance of a food web generally decreases with increasing species richness to remain a stable system; Pimm, 1982; Strong, 1988; Lawton, 1989), but differ in other details (e.g. streams have shorter food chains than lake ecosystems; Vander-Zanden and Fetzer, 2007). Differences are mainly based on the fact that populations and community structures are influenced by a host of biotic and abiotic factors that act simultaneously (Carpenter et al., 1987). Nowadays, there is evidence that food web connections are biologically mediated by "bottom-up" and "top-down" controls (Smith, 1969; Carpenter et al., 1985; Wetzel, 2002; Hulot et al., 2014); but also specific environmental attributes are defining not only the communities living in the aquatic ecosystems, but also the activities of organisms and the transformation of energy and matter (Lamper and Sommer, 1997). Then, we should expect relative roles of different ecological forces to vary among biological systems and even within the same system when environmental heterogeneity is taken into account (Dunson and Travis, 1991). For example, in lakes, the spatial community structure is defined, mostly, by two zones, the pelagic and benthic habitats; in rivers the flow makes them essentially different, with concepts of nutrient spiraling (Newbold et al., 1981) and river continuum (Vannote et al., 1980) as main concerns defining the lotic food web structure.

A complete understanding of ecosystems also requires the identification of biogeochemical processes that define nutrient dynamics: the ecosystem functioning. Among all the nutrients available in Earth, nitrogen and phosphorous (P) are of primary interest to limnologists and biogeochemists, because both elements potentially limit primary production, controlling the dynamics of aquatic ecosystems (Grimm and Fisher, 1986; Robinson, 2001; Wetzel, 2001; Elser et al., 2007).

A.2 NITROGEN PROCESSES IN AQUATIC ECOSYSTEMS

Nitrogen is as a key element in aquatic systems, and the knowledge of its forms and transformations are important issues to understand its global cycle (Dodds, 2002). Most N in the biosphere is present as N_2 gas in the atmosphere (78% of atmosphere is N_2), which can be introduced into the water ecosystems via precipitation, dry deposition and fixation (Wetzel, 2001; Fig. A.1).

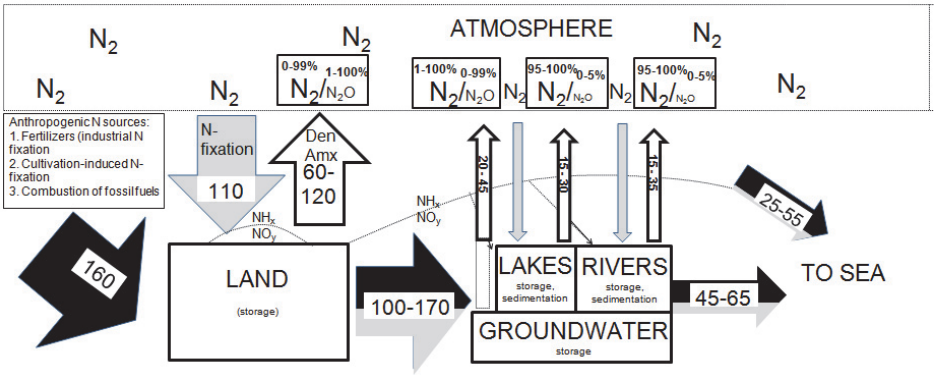

Fig. A.1: Modern global nitrogen cycle (Tg N $year^{-1}$). The proportions of naturally and anthropogenically fixed N in the flow and transport of N is depicted with solid grey and black arrows, respectively. Microbial reactions removing N in gaseous forms (N_2 and N_2O), are depicted with black outlined arrows (taken from Rissanen, 2012).

Apart from N_2 inputs, N can also income to inland waters via surface runoff and groundwater drainage resulting in a diverse pool of N components: dissolved gasses (molecular N_2 and nitrous oxide N_2O), dissolved inorganic compounds (ammonium -NH_4, nitrite -NO_2- and nitrate -NO_3), and a large number of dissolved and particulate organic forms (amino acids, proteins and humic compounds). These chemicals are transformed mainly through biotic activities, creating the aquatic nitrogen cycle (Duff and Triska, 2000). The form in which N is presented has a range of ecological consequences, including

the degree of N mobility, its biological availability and even its toxicity (Stanley and Maxted, 2008).

The N cycle in aquatic ecosystems consists of a balance between nitrogen inputs to and nitrogen losses (Wetzel, 2001). Some organisms have the capacity to assimilate N_2 and transform it to NH_4 and NO_3 which are quickly assimilated by primary producers and other bacterial growth, and integrated into organic compounds (biological nitrogen fixation; Vitousek et al., 2002). As a result of microbial activity, NH_4^+ can be oxidized to NO_2/NO_3 via nitrification (Ward, 1996; Bartrons, et al., 2010; Auguet et al., 2011, 2012), while by reverse processes, NO_2/NO_3 can be converted to NH_4 via dissimilatory nitrate reduction (DNRA; Schmid et al., 2007). By these routes, nitrogen remains in biologically available forms for organism uptake. However, there are also other pathways that remove N compounds from the ecosystems, producing N_2O and N_2 via anaerobic oxidation of NH_4 (Anammox; Penton et al., 2006; Schmid et al., 2007; Yosinaga et al., 2011) and by the reduction of NO_3^- during the denitrification (Francis et al., 2007; Findlay et al., 2011). Although all these processes are mediated by the appearance of different microbial communities, physical and chemical parameters as temperature, oxygen content, pH and redox conditions which are force drivers allowing these transformations (e.g. depending on the redox conditions, organisms will utilize different oxidized materials as electron acceptors with a general order from O_2, NO_3, SO_4^2 to CH_4; Kendall and McDonnell, 1998). Consequently, these components and processes in aquatic ecosystems are highly variable seasonally and spatially (Wetzel, 2001), taking place in every ecosystem compartment (e.g. water column, sediment, sediment-water interface and macrophyte root zone; Rysgaard et al., 1993; Piña-Ochoa and Alvarez-Cobelas, 2006; Epstein et al., 2012).

The direct simultaneous measurement of all the dynamics within the complex N cycle, can be very challenging. Different approaches have contributed to the calculation of individual processes. These approaches have often limited the integration of all the biogeochemical processes, which is critical for ecosystem function (Middelburg, 2014). By conducting Stable Isotope Analyses (SIA), the temporal stability of stable isotopes (SI) can be exploited to use them as biogeochemical tracers, allowing measurement of simultaneous ecological and biogeochemical processes at whole-ecosystem scale, integrating small-scale variability to give an effective indication of catchment-scale processes (Kendall and Doctor, 2003; Fry, 2006; Bouillon et al., 2012).

A.3 STABLE ISOTOPES AS TRACERS IN LIMNOLOGICAL STUDIES

Concepts, measurement and terminology

Isotopes (meaning they all occupy the same -iso- place -topos- in the periodic table) are forms of an element that differ in the number of neutrons in the nucleus and that organisms cannot directly detect because it exists at the atomic level (Fry, 2006). Having an extra neutron, therefore the same atomic number but different atomic mass, does make only a very slight difference in some reactions, making the heavier isotope the slower (this reaction is named fractionation process). Stable isotopes, which do not appear to decay over time, comprise less than 10% of all the known isotopes and are naturally present and circulating in natural systems (283 stables isotopes; see Clayton, 2003 for further information). In this thesis we only focus on nitrogen and carbon (C) elements, and both are composed of one abundant (^{14}N and ^{12}C) and one of the relatively minor abundance isotopes (^{15}N and ^{13}C) (Table A.1, Fry 2000;

Clayton 2003). The low abundance of these heavier isotopes provides the opportunity to use them as natural dyes tracking the circulation of the elements at many levels, from individual microbes to whole-ecosystem scale, in biochemical, biological and environmental studies (Unkovich et al., 2001; Fry, 2006).

Table A.1: Isotopes composition of international references standards. H and L indicate heavy and light isotope components, respectively. Absolute abundance of isotopes are reported as the ratio H/L between high mass and low mass isotopes. Values are taking from Fry, 2001.

	Ratio, H/L	**Value, H/L**	**% H**	**% L**
Air (AIR)	$^{15}N/^{14}N$	0.00363	0.366	99.63
PeeDee Belemnite (PDB)	$^{13}C/^{12}C$	0.01118	1.105	98.89

Absolute abundance of isotopes are reported as the ratio (R) between the less abundant -always the heavier- and the more abundant -the lighter- (Table A.1). However, because isotopic differences between natural samples usually occur beyond the third significant number of this ratio, most ecological studies express isotopic abundances using δ notation, meaning part per thousand differences from a standard (Peterson and Fry, 1987):

$$\delta=[(R_{sample}/R_{standard})-1] *1000 \quad \text{(Eq. A. 1)}$$

$$R_{sample}= [(\delta/1000) + 1] * R_{standard} \quad \text{(Eq. A. 2)}$$

Most of the $\delta^{13}C$ and $\delta^{15}N$ values for natural samples range between -100 and +50 ‰, which increases in δ values denote increases in the amount of the heavy isotope component (Fig. A.2). Large natural abundance δ variation from -100 to +100 correspond to only slight variations in percent heavy isotope**.**

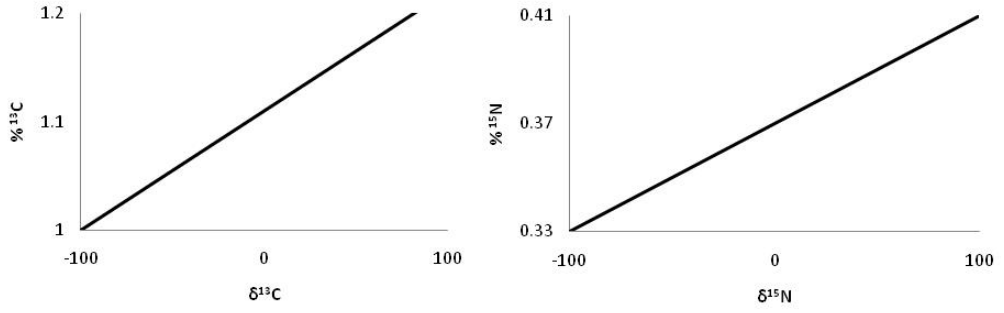

Fig. A.2: Linear relationships of a) carbon and b) nitrogen heavy isotope contents to δ values.

Based on the stable isotope theory, there is an overall circulation with processes generating labeled substances via fractionation that then mix and recombine creating new products with different isotope signals (δ), resulting from physical, chemical and biological reactions (Fry, 1991; Kendall and Doctor, 2003). The explanation behind is a bit more complex than this, but as a simplification we can say that fractionation and mixing, together, control isotope cycling and circulation. As a result, the compounds develop unique isotopic compositions that indicate their source or the processes that formed them (for further information see Peterson and Fry, 1987 and Fry, 2006). Then, approaches based on stable isotopes have the potential to provide more detailed insight on food web relationships and nutrient dynamics in aquatic ecosystems (Peterson and Fry, 1987; Fry, 2006; Layman et al., 2007; Newsome et al., 2007, Bouillon et al., 2012; Soto et al., 2013; Middelburg, 2014).

$\delta^{15}N$ and $\delta^{13}C$ as tracers in aquatic ecosystems: from natural abundances approaches to in situ labeling experiments at whole ecosystem-scale

Because $\delta^{15}N$ and $\delta^{13}C$ measurements can be performed on inorganic substrates, detrital and living organic matter, it provides a unique opportunity to use the same currency for single-celled and large organisms, dead organic matter and inorganic compounds (Middelburg, 2014). The massive amount of N in the biosphere shows $\delta^{15}N$ values near the 0‰ (from −10 to +10‰; Peterson and Fry, 1987) with no overall or very slight isotope fractionation (Dodds, 2002; Fry, 2006). However, larger isotope contrasts might be expected between and within freshwaters with large possible fractionations, where $\delta^{15}N$ values reflect the isotopic fractionations resulting from reactions such as uptake and assimilation in food webs, nitrification or denitrification (Peterson and Fry, 1987; Ohte et al., 2010). The majority of the reactions and processes almost always discriminate between isotopes and generally favors the incorporation of ^{14}N over ^{15}N, resulting in ^{15}N enrichment of the substrate and ^{15}N depletion of the product (e.g., the faster loss of ^{14}N than ^{15}N during particulate N decomposition results in $\delta^{15}N$ increases of 5 to 10‰ with increasing depth in the sediment; Kendall and Doctor, 1998). Many of these biological processes consist of a number of steps (e.g. nitrification: organic N $\rightarrow NH_4 \rightarrow NO_2 \rightarrow NO_3$), where each step has the potential for ^{15}N discrimination and the overall fractionation is highly dependent on environmental condition including number and type of intermediate steps, sizes of pools (e.g. in N-limited systems, the fractionations during the nitrification are minimal; Kendall and Doctor, 1998). Fixation of atmospheric N_2 by the organisms commonly produces organic materials with $\delta^{15}N$ values slightly less than 0‰, therefore low $\delta^{15}N$ values in organic matter are often cited as evidence for N_2 fixation (Fogel and Cifuentes, 1993; Kendall, 1998). In systems where the $\delta^{15}N$ associated to the

phytoplankton has different signatures than terrestrial vegetation, it works as source markers to distinguish between autochthonous and allochthonous organic matter (Armengol et al., 2012). Thus, the use of ^{15}N as tracer in aquatic ecosystems, to identify and quantify biogeochemical fluxes of material and energy is possible.

The dual combination of $\delta^{13}C$ and $\delta^{15}N$ natural abundances broadens the possibilities for more ecological applications, including the elucidation of energy and matter flows in food webs and the temporally and spatially integrated construction of dietary pathways (DeNiro and Epstein, 1978; Fry, 1991; Bearhop et al., 2004; Mildellburg, 2014). DeNiro and Epstein (1981) showed that measurements of $\delta^{15}N$ of a consumer's tissues are usually higher than those of its diet, and the magnitude of the difference is relatively consistent among organisms. Consequently, $\delta^{15}N$ of aquatic organisms becomes a powerful tool for estimating their trophic positions (Minagawa and Wada, 1984; Cabana and Rasmussen, 1996; Vander Zanden and Rasmussen, 2001; Vanderklift and Ponsard, 2003). $\delta^{13}C$ varies substantially among primary producers with different photosynthetic pathways (e.g. C_4 plants have $\delta^{13}C$ values more similar to atmospheric CO_2, -20 to -10 ‰; C_3 plants: -33 to -24; O´Leary, 1988), but change little with trophic transfers (0-1‰; DeNiro and Epstein, 1981; Peterson and Fry, 1987; Post, 2002; Inger and Bearhop, 2008). Therefore, $\delta^{13}C$ are applied to determine original sources of dietary carbon (Cole et al., 2006). In addition to estimating vertical position in a web and quantifying proportional contributions of source pools, community metrics based on $\delta^{13}C$ and $\delta^{15}N$ signatures provide more general depictions of food-web structure (Layman et al., 2012). Both isotopes are commonly graphed in a bi-plot to depict niche space, as a potential way to investigate ecological niches (Newsome et al., 2007). Besides, the isotope data can also be incorporated to

models and a series of spatial metrics to detail more information regarding trophic structure (e.g., trophic diversity and species packing within communities; Layman et al., 2007). As a result, the study of $\delta^{13}C$ and $\delta^{15}N$ natural abundances have dramatically improved our understanding of numerous aspects of trophic structure, providing new insights into food-chain length (Post, 2002) and niche variation (Newsome et al., 2007; Martinez del Rio et al., 2009; Semmens et al., 2009; Jackson et al., 2012). However, in some circumstances, this approach shows important limitations that make it poorly effective to characterize the structure and functioning of aquatic ecosystems.

One of the main constraints resides in isotopic signals of different pools that are very similar (i.e. sources are not isotopically distinguished; Peterson et al., 1985, Raikow and Hamilton, 2001). While evaluations of the potential contribution of a specific N source that has a unique isotopic signature are often successful (Minagawa and Wada, 1984; Middelburg, 2014), interpretations of the contributions of different N sources or more complex processes generally needed confirmation by some independent non-isotopic method or isotope tracer methods (Hall et al., 2009a). Deliberate stable isotope addition methods combine the potential of isotope ecological theory with nutrient enrichment methods, while maintaining natural hydrologic and biogeochemical gradients (Peterson and Fry, 1987; Tobias et al., 2003; Middelburg, 2014). The main advantage of using this approach in a whole-ecosystem resides in providing cuasi-actual rate estimates rather than potential rates, and avoiding the limitations of mesocosms or laboratory experiments. The use of whole-ecosystem in situ ^{15}N additions have enabled limnologist to study N cycling and food web structure in a variety of ecosystems: streams (Peterson et al., 1997; Hall et al., 1998; Mulholland et al., 2000; Ashkenas et al., 2004), lakes (Armengol et al., 2012; Hadwen and Bunn, 2005), and

estuaries and marshes (Holmes et al., 2000; Tobias et al., 2003; Gribsholt, et al., 2009).

Overall, either natural abundance or *in situ* deliberate addition approaches of ^{15}N and ^{13}C stable isotopes, applied in aquatic ecosystems permit us to study specific processes, including community structure and biogeochemical rates. Moreover, the combination of SIA with other ecological methods, such DNA-based approaches offer an even further exploration of the ecosystem functioning.

Los ecosistemas acuáticos continentales (principalmente lagos, embalses y ríos), aunque ocupan sólo una pequeña parte de la superficie terrestre, tienen una gran importancia en términos de significado ecológico y como recurso natural de bienes y servicios para el ser humano (Lamper y Sommer, 1997; Wetzel, 2001; Carpenter et al., 2011; Cosgrove et al., 2014). Sin embargo, durante el siglo pasado, estos ecosistemas han sido drásticamente modificados por perturbaciones, a escala global y local, experimentando un ritmo acelerado de degradación (ej. la eutrofización, pérdida de biodiversidad y la contaminación; Wetzel, 2002; Shurin et al., 2012). Una de las mayores presiones ejercidas en los ecosistemas acuáticos es la alteración del ciclo del N, pudiéndose haber superado ya los umbrales de recuperación en muchos casos. (Rochström et al., 2009). Por ello, y con el objetivo de poder predecir y contrarrestar las actuales y futuras alteraciones en estos recursos naturales, es cada vez más importante la adquisición de un conocimiento integral sobre la ecología de estos ecosistemas (Wetzel, 2001; Saunders y Kalff, 2001; Dodds, 2002, 2013; Mantyka-Pringle et al., 2014).

Con el objetivo principal de estudiar en profundidad los procesos y dinámicas relativos a la ecología de los ecosistemas acuáticos, los limnólogos, en muchas ocasiones, reducen el significado global de ecosistema a unidades más manejables, valiéndose de simplificaciones y desarrollando generalizaciones (Jopp et al., 2011). En el estudio de estos ecosistemas, los conceptos de estructura trófica y dinámica de nutrientes se han desarrollado como herramienta para caracterizar a las comunidades e identificar relaciones y redes entre especies, definir compartimentos bióticos, y para estudiar los vínculos entre variables ambientales y procesos funcionales (Pimm, 2002; Dodds, 2002; Mutshinda y O'Hara, 2011; Cardinale, 2011; Thompson et al., 2012;. Abrantes et al., 2014). Estos conceptos son características claves de los

ecosistemas y, desde hace muchos años, han sido definidos como herramientas fundamentales en el estudio de la ecología acuática (Wetzel, 2002; Glibert et al., 2011; Sardans et al., 2011).

A.1 ESTRUCTURA DE LA RED TRÓFICA

Los ecosistemas están estructurados en el sentido de que están compuestos de diferentes elementos, bióticos y abióticos, y de que éstos se disponen formando una red de interacciones con patrones definidos (Margalef, 1963; Keller y Golley, 2000). Durante décadas, se han desarrollado varias metodologías para estudiar diferentes aspectos y atributos de la estructura de los ecosistemas acuáticos, como la riqueza de especies (ej. el índice de Shannon-Wiener; Shannon y Weaver, 1948), la integridad biótica (ej. el índice de integridad biótica IBI; Karr, 1981) o la caracterización de los recursos basales (ej. el rol de la materia alóctona *vs*. autóctona; Sterner et al., 1997). Entre las interacciones más importantes dentro de un ecosistema se encuentran las de carácter trófico: relaciones y redes que se han utilizado de manera generalizada para la evaluación y caracterización de la estructura de los ecosistemas acuáticos (Lindemans, 1942; Scheffer., 1998; Vander Zanden et al, 2003; Abrantes et al., 2014;. Middelburg., 2014).

Por la tremenda complejidad de los ecosistemas naturales, el estudio y la definición de estructuras y dinámicas dentro de las redes tróficas es, en la mayoría de los casos, un gran desafío intelectual (Winemiller y Layman, 2005). Entre las herramientas más utilizadas para este fin se incluyen la evaluación de la diversidad trófica, el estudio del nicho ecológico, y el cálculo del grado de empaquetamiento y de las conexiones entre las especies dentro del ecosistema (Layman et al., 2007; Newsome et al., 2007;. Schmidt et al., 2007). En la

mayoría de los casos, los descriptores tróficos de se basan, principalmente, en el número total de especies, como es caso del análisis de las relaciones tróficas (fuente-consumidor) o la longitud de la cadena trófica (May, 1986; Lawton, 1989; Warren, 1990). Cada uno de estos enfoques presenta ventajas y debilidades, teniendo una mayor aplicabilidad dependiendo de las características especificas que requiera el estudio en cuestión. No obstante, cuantos más descriptores y atributos tróficos se obtengan, la aproximación de las interacciones y procesos que engloban la estructura de los ecosistemas acuáticos será más detallada.

Como resultado de la aplicación de estos descriptores, hoy sabemos que, en lo relativo a la estructura trófica, la mayoría de los ecosistemas acuáticos comparten algunas generalidades y patrones (por ejemplo, la conectancia de una red trófica, generalmente, disminuye con el aumento de la riqueza de especies con el fin de mantener la estabilidad del sistema; Pimm, 1982; Strong, 1988; Lawton, 1989); sin embargo, difieren en otras muchas características que son más propias de cada ecosistema (ej. los sistemas lóticos suelen albergar comunidades con cadenas tróficas más cortas que los ecosistemas lénticos; Vander-Zanden y Fetzer, 2007). Estas diferencias se basan, en gran medida, en la influencia que ejercen, de forma simultánea, los factores bióticos y abióticos sobre las poblaciones y comunidades (Carpenter et al., 1987). Actualmente, existen evidencias suficientes para pensar que la estructura que define las redes tróficas de estos ecosistemas, están controladas bióticamente por los efectos de "botton-up" y "top-down" (Smith, 1969; Carpenter et al., 1985, Wetzel, 2002;. Hulot et al., 2014), además de por las variables ambientales que caracterizan a cada ecosistema (Lamper y Sommer, 1997). Por ello, es razonable pensar que las comunidades bióticas se estructurarán de forma característica, acorde con los factores bioticos y abióticos, dando como resultado una heterogeneidad

espacial no solo entre ecosistemas, sino también dentro de un mismo ecosistema (Dunson y Travis, 1991). Por ejemplo, en el caso de los lagos, la estructura de las comunidades se define, principalmente, por dos hábitats: el pelágico y el bentónico; mientras que en los ríos, el flujo de agua condiciona el concepto de espiral de nutrientes y de continuidad de los principales atributos que definen la estructura trófica de los ecosistemas lóticos (Vannote et al., 1980; Newbold et al., 1981).

No obstante, para poder profundizar y llegar a una comprensión completa sobre cómo se estructuran los organismos y comunidades dentro de los ecosistemas, es fundamental la identificación y el estudio de los procesos biogeoquímicos que definen la dinámica de nutrientes en estos ecosistemas. Entre todos los nutrientes disponibles en la Tierra, el nitrógeno, el carbono (C) y el fósforo (F) son, para limnólogos y biogeoquímicos, los que mayor interés suscitan, principalmente porque ambos elementos son potencialmente limitantes de la producción primaria en los ecosistemas acuáticos (Grimm y Fisher, 1986; Robinson, 2001; Wetzel, 2001; Elser et al., 2007).

A.2 DINÁMICA DEL NITRÓGENO EN LOS ECOSISTEMAS ACUÁTICOS

El N es reconocido como un elemento clave dentro de los sistemas acuáticos, y el estudio de sus compuestos y de los procesos de transformación, cobran una importancia vital a la hora de poder entender su ciclo global (Dodds, 2002). De forma natural, la mayoría del N está presente en forma gaseosa (78% de la atmósfera es N_2), y éste puede llegar a formar parte de los ecosistemas acuáticos a través de procesos de precipitación, deposición seca y fijación (Wetzel, 2001; Fig. A.1).

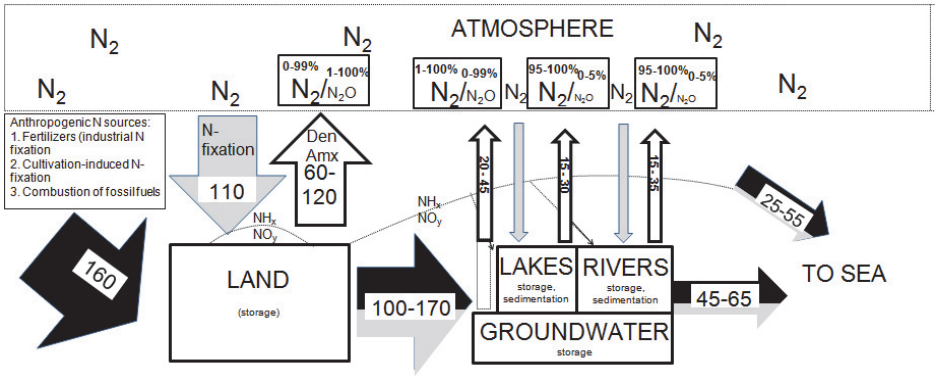

Fig. A.1: Ciclo global del nitrógeno (Tg N año^{-1}). Las cantidades de N en formas de flujos naturales y antrópicos se representan con flechas de color gris y negro sólido, respectivamente. Las reacciones microbianas que eliminan el N de los ecosistemas acuáticos vía forma gaseosa se representan con flechas con contorno marcada de color negro (tomado de Rissanen, 2012).

Aparte de las entradas de N por vía atmosférica, los ecosistemas acuáticos también reciben aportes de N a través de la escorrentía superficial y el drenaje de las aguas subterráneas, lo que provoca la aparición en sus aguas de un diverso grupo de compuestos nitrogenados: gases disueltos (nitrógeno molecular -N_2- y óxido nitroso -N_2O-), elementos inorgánicos disueltos (amonio -NH_4^+-, nitrito -NO_2^-- y nitrato -NO_3^--), y un gran número de compuestos orgánicos disueltos y particulados (aminoácidos, proteínas y compuestos húmicos). La presencia y abundancia de cada uno de estos compuestos nitrogenados en los sistemas naturales tiene importantes consecuencias ecológicas, incluyendo el grado de movilidad del N, su disponibilidad biológica, e incluso su toxicidad (Stanley y Maxted, 2008). Mediante diferentes actividades bióticas, principalmente microbianas, estos compuestos son transformados de unos a otros, creando el ciclo del nitrógeno (Duff y Triska, 2000).

El ciclo de N consiste en el balance entre los aportes y las pérdidas de N en un ecosistema (Wetzel, 2001). Algunos organismos tienen la capacidad de asimilar N_2 y transformarlo a NH_4^+ y NO_3^-, compuestos que pueden ser

incorporados a los productores primarios y comunidades microbianas y, por tanto, transformados, a compuestos orgánicos (fijación biológica del nitrógeno; Vitousek et al., 2002). Como resultado de la actividad microbiana, el NH_4^+ también puede ser oxidado a NO_2^- / NO_3^- mediante el proceso de nitrificación (Ward, 1996; Bartrons, et al., 2010; Auguet et al., 2011, 2012), mientras que a su vez, y de forma simultánea, NO_2^- / NO_3^- pueden ser convertidos a NH_4^+ por medio de un proceso de reducción desasimilador del nitrato (DNRA; Schmid et al., 2007). Estas rutas enzimáticas permiten mantener el N dentro del ecosistema acuático, en compuestos que son asimilables por los organismos.

Otros procesos, sin embargo, potencian la eliminación de compuestos nitrogenados fuera del ecosistema acuático, principalmente, mediante la producción de gases de N_2O y N_2 durante el proceso de reducción de NO_3^- o desnitrificación (Francis et al., 2007; Findlay et al., 2011) y el de la oxidación anaerobia de NH_4^+ (Anammox; Penton et al, 2006; Schmid et al., 2007; Yosinaga et al., 2011). Todos estos procesos están mediados por la presencia de comunidades microbianas específicas, pero variables físico-químicas como la temperatura, el contenido de oxígeno, y las condiciones de pH y redox son factores importantes que definen, en gran parte, estas transformaciones (por ej. dependiendo de las condiciones redox, los organismos utilizarán diferentes compuestos como aceptores de electrones, según el siguiente orden: O_2, NO_3^-, SO_4^{2-}, y CH_4; Kendall y McDonnell, 1998). En consecuencia, estos procesos, así como las proporciones de cada compuesto de N, pueden ser muy variables inter e intra ecosistemas acuáticos, a escala temporal y espacial (Wetzel, 2001), produciéndose incluso grandes diferencias entre compartimentos de un mismo ecosistema (por ej. entre la columna de agua, el sedimento, la interfase sedimento-agua y la zona de raíces; Rysgaard et al., 1993; Piña-Ochoa y Álvarez-Cobelas, 2006; Epstein et al., 2012).

En los ecosistemas naturales, la evaluación directa y el estudio simultáneo de todas estas transformaciones son tareas complejas de abordar. Es por ello que, habitualmente los estudios se han enfocado en el cálculo de los procesos de forma individual, limitando, a menudo, la integración de todos los procesos biogeoquímicos dentro de un ecosistema (Middelburg, 2014). Como una posible alternativa para evitar estas limitaciones, el análisis de los isótopos estables (SIA) surge como una herramienta que permite estudiar los procesos ecológicos y biogeoquímicos de forma simultánea en todo el ecosistema (Kendall y Doctor, 2003; Fry, 2006; Bouillon et al., 2012).

A.3 LOS ISÓTOPOS ESTABLES COMO TRAZADORES EN ESTUDIOS LIMNOLOGICOS

Conceptos, medición y terminología

Los isótopos (que significa que todos ocupan el mismo -iso- lugar -topos- en la tabla periódica) son formas de un elemento químico que difieren en el número de neutrones en el núcleo y que no pueden ser detectado directamente por los seres vivos, ya que existen en el nivel atómico (Fry, 2006). El factor de tener un neutrón extra, por lo tanto, el mismo número atómico pero diferente masa atómica, provoca pequeñas diferencias entre los isótopos de un mismo elemento y en la transformación de los mismos, básicamente, debido a que el isótopo más pesado es más lento en las reacciones físico-químicas (esta reacción se denomina fraccionamiento). Los isótopos estables engloban menos del 10% de todos los isótopos conocidos, están presentes y circulando de forma natural en los sistemas naturales y, al contrario que los isotopos radiactivos, no se degradan con el tiempo (actualmente hay 283 isótopos estables conocidos; para más información consultar Clayton, 2003). Esta tesis se centra,

únicamente, en dos isótopos estables del N y el C, siendo uno abundante y más conocido (^{14}N y ^{12}C) y uno relativamente de menor abundancia (^{15}N y ^{13}C) (Tabla A.1, Fry, 2000; Clayton, 2003).

Tabla A.1: Abundancia de los isótopos estables de N y C y los estándares internacionales de referencia. H y L indican el isótopos pesado y ligero, respectivamente. La abundancia absoluta de isótopos se señala como la relación H/L (en Fry, 2001).

	Ratio, H/L	**Valor, H/L**	**% H**	**% L**
Aire (AIR)	$^{15}N/^{14}N$	0.00363	0.366	99.63
PeeDee Belemnite (PDB)	$^{13}C/^{12}C$	0.01118	1.105	98.89

La baja abundancia de los isótopos más pesados ofrece la oportunidad de utilizarlos como trazadores naturales para seguir la circulación de esos elementos en estudios bioquímicos, biológicos y ambientales, a diferentes escalas, desde comunidades microbianas hasta la escala más holística de todo un ecosistema (Unkovich et al., 2001; Fry, 2006). La abundancia absoluta de los isótopos se expresa como la relación (R) entre los menos abundantes, más "pesados", y los más abundantes, más "ligeros" (Tabla A.1). Sin embargo, debido a que las diferencias isotópicas entre muestras naturales ocurren generalmente a niveles numéricos de magnitud muy pequeña, la mayoría de los estudios expresan las abundancias isotópicas utilizando la denotación delta (δ), es decir, una parte por mil diferencias (Peterson y Fry, 1987):

$$\delta=[(R_{sample}/R_{standard})-1] *1000 \quad \text{(Eq. A.1)}$$

$$R_{sample}= [(\delta/1000) + 1] * R_{standard} \quad \text{(Eq. A.2)}$$

La mayoría de los valores de $\delta^{13}C$ y $\delta^{15}N$ para muestras naturales oscilan entre -100 y 50 ‰, donde incrementos en los valores δ denotan aumentos en la cantidad del componente isotópico más pesado. Variaciones naturales de δ entre -100 y +100 corresponde con ligeras variaciones en el porcentaje de isótopo pesado (Fig. A.2).

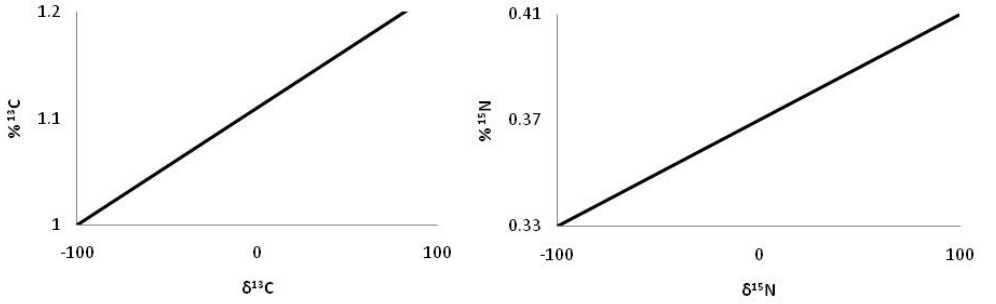

Figura A.2: Contenido de los isótopos pesados de C y N en relación a los valores de delta (δ).

Siguiendo la teoría general de los isótopos estables: en la naturaleza, las reacciones físicas, químicas y biológicas producen una circulación continua de los isótopos, donde procesos, como el fraccionamiento, generan sustancias marcadas isotópicamente, que luego, a su vez, se mezclan y re-combinan creando nuevos productos, todos con diferentes señales isotópicas definidas (δ) (Fry, 1991; Kendall y Doctor, 2003). La explicación detrás de todos estos procesos es un poco más compleja, pero a modo de simplificación, podemos asumir que, juntos, el fraccionamiento y el proceso de mezcla controlan el ciclo y las abundancias de los isótopos ^{15}N y ^{13}C en los sistemas naturales. Como resultado, los compuestos de N y C tienen proporciones isotópicas únicas, que indican su origen y dan información sobre los procesos que los formaron (para más información véase Peterson y Fry, 1987; Fry, 2006). De esta manera, los

isótopos ^{15}N y ^{13}C tienen el potencial de proporcionar una visión más detallada de las relaciones tróficas y de la dinámica de nutrientes en los ecosistemas acuáticos (Peterson y Fry, 1987; Fry, 2006; Layman et al., 2007; Newsome et al., 2007., Bouillon et al., 2012; Soto et al., 2013; Middelburg, 2014).

$\delta^{15}N$ y $\delta^{13}C$ como trazador en los ecosistemas acuáticos: abundancia natural y experimentos de adición *in situ*

Debido a que las medidas de $\delta^{15}N$ y $\delta^{13}C$ se pueden realizar tanto en sustratos inorgánicos y detríticos, como en la materia orgánica viva, estas proporcionan una oportunidad única para utilizar el mismo trazador en diferentes compartimentos (Middelburg, 2014). El N de la atmosfera tiene valores de $\delta^{15}N$ cercanos al 0 ‰ (del -10 al + 10 ‰; Peterson y Fry, 1987), con fraccionamientos, en general, muy leves (Dodds, 2002; Fry, 2006). Sin embargo, en los ecosistemas acuáticos se observan grandes contrastes isotópicos en los diferentes compuestos nitrogenados, donde valores de $\delta^{15}N$ reflejan los fraccionamientos resultantes de reacciones biogeoquímicas (por ej. asimilación dentro de las redes tróficas o procesos de nitrificación y desnitrificación; Peterson y Fry, 1987; Ohte et al., 2010).

La mayoría de las reacciones y procesos discriminan entre isótopos, favoreciendo, generalmente, la incorporación de ^{14}N frente a ^{15}N, teniendo como resultado sustratos o fuentes enriquecidos en ^{15}N y productos con menor abundancia de ^{15}N (durante los procesos de descomposición de compuestos de N particulado, la pérdida más rápida de ^{14}N frente a ^{15}N, provoca incrementos en los valores de $\delta^{15}N$ de entre 5 a 10 ‰ con respecto a la profundidad del sedimento; Kendall y Doctor, 1998). Muchos de los procesos biológicos consisten en una serie de sub-transformaciones (por ej. la nitrificación: N

orgánico $\rightarrow NH_4 \rightarrow NO_2 \rightarrow NO_3$), donde cada episodio tiene un potencial de discriminación frente al ^{15}N y en donde el fraccionamiento global de todo el proceso depende de factores tales como el número de pasos intermedios y las concentraciones que presentan cada producto (Kendall y Doctor, 1998). Por lo tanto, es posible usar el ^{15}N como trazador para identificar y cuantificar los flujos biogeoquímicos dentro de los ecosistemas acuáticos. La fijación del N_2 atmosférico por los organismos crea materia orgánica con valores de $\delta^{15}N$ cerca del 0 ‰; por lo tanto, valores bajos de $\delta^{15}N$ encontrados en la materia orgánica son a menudo citados como evidencias de fijación del N_2 (Fogel y Cifuentes, 1993; Kendall y 1998). En los ecosistemas acuáticos donde el fitoplancton tiene asociado una firma isotópica de $\delta^{15}N$ diferente a los valores de la vegetación terrestre, ^{15}N actúa como marcador para distinguir entre la materia orgánica autóctona y alóctona (Armengol et al., 2012).

El estudio simultáneo de las abundancias naturales de $\delta^{15}N$ y $\delta^{13}C$ amplía las posibilidades de las aplicaciones ecológicas de estos isótopos, incluyendo el esclarecimiento de los flujos de energía y materia en las redes tróficas y el estudio integral de las escalas temporales y espaciales de las rutas tróficas (DeNiro y Epstein, 1978; Fry, 1991; Bearhop et al., 2004; Mildellburg, 2014). Los valores de $\delta^{15}N$ medidos en los tejidos de los consumidores son generalmente más altos que los de su dieta y la magnitud de dicha diferencia es relativamente constante entre los organismos (DeNiro y Epstein, 1981). Como consecuencia, los valores de $\delta^{15}N$ en los organismos acuáticos pueden ser usados como una herramienta para la estimación de las posiciones tróficas (Minagawa y Wada, 1984; Cabana y Rasmussen, 1996; Vander Zanden y Rasmussen, 2001; Vanderklift y Ponsard, 2003). Por el contrario, los valores de $\delta^{13}C$ varían poco durante las transferencias entre niveles tróficos (0-1 ‰; DeNiro y Epstein, 1981; Peterson y Fry, 1987; Post, 2002; Inger y Bearhop,

2008;). Sin embargo, $\delta^{13}C$ si varía sustancialmente entre los productores primarios: las plantas C4 tienen valores de $\delta^{13}C$ más cercanos al $\delta^{13}C$ del CO_2 atmosférico (-20 a -10 ‰), mientras que las plantas C3 presentan valores más empobrecidos en ^{13}C (-33 a -24) (O'Leary, 1988). Por lo tanto, $\delta^{13}C$ se usa para determinar las fuentes originales de carbono en la dieta (Cole et al., 2006). Generalmente, en el estudio de cadenas tróficas y nichos ecológicos, los datos isotópicos de las especies o comunidades se representan en gráficas de biplot (en la forma de $\delta^{15}N/\delta^{13}C$; Newsome et al., 2007). Además de estimar la posición vertical en la red trófica e identificar y cuantificar el origen de las fuentes, los valores de $\delta^{13}C$ y $\delta^{15}N$ incorporados en métricas o modelos a escala de comunidad proporcionan descripciones más generales de la estructura trófica de los ecosistemas acuáticos (diversidad trófica y empaquetamiento de especies dentro de las comunidades; Layman et al., 2007; Layman et al., 2012). Como resultado, el uso los valores de abundancia natural de $\delta^{13}C$ y $\delta^{15}N$ en los estudios de los ecosistemas acuáticos ha incrementado enormemente el conocimiento de numerosos aspectos relacionados con la estructura trófica, proporcionando nuevas claves, entre otros aspectos, sobre la longitud de las redes tróficas (Post, Pace y Hairston, 2000) y la variación de nicho ecológico (Newsome et al., 2007; Martínez del Río et al., 2009; Semmens et al, 2009; Jackson et al., 2012). Sin embargo, en algunas circunstancias, esta herramienta muestra importantes limitaciones, lo que provoca que sea poco efectiva para caracterizar ciertos aspectos del funcionamiento de los ecosistemas acuáticos.

Una de las principales limitaciones ocurre cuando los valores isotópicos (δ) de diferentes compuestos o fuentes son muy similares (es decir, las fuentes no se pueden distinguir mediante sus valores isótopos; Peterson et al., 1985; Raikow y Hamilton, 2001). En estos casos, las interpretaciones de las contribuciones de las diferentes fuentes o procesos necesitan de una

confirmación por otros medios no isotópicos, o mediante la adición de isótopos como trazadores (Hall et al., 2009). La metodología de adición de isótopos estables combina el potencial de la teoría isotópica y los métodos de enriquecimiento de nutrientes, con la ventaja de que se mantienen las condiciones hidrológicas y biogeoquímicas naturales durante todo el experimento (Peterson y Fry, 1987; Tobias et al., 2003; Middelburg, 2014). La principal ventaja de utilizar este enfoque a escala ecosistémica reside en que proporciona estimaciones de tasas cuasi-reales en lugar de tasas potenciales, minimizando las limitaciones de extrapolación que tienen los estudios en mesocosmos o los experimentos de laboratorio. De este modo, la técnica adición *in situ* de ^{15}N a escala de todo el ecosistema acuático ha permitido a los limnólogos estudiar en profundidad el ciclo del N y la estructura de la red trófica en una gran variedad de ecosistemas acuáticos: ríos y arroyos (ej. Peterson et al., 1997; Hall et al., 1998; Mulholland et al., 2000; Ashkenas et al., 2004), lagos (Armengol et al., 2012; Hadwen y Bunn., 2005), estuarios y marismas (Holmes et al., 2000; Tobias et al., 2003; Gribsholt, et al., 2009).

El uso de los isótopos estables ^{15}N y ^{13}C, por medio de medidas de su abundancia natural y de adiciones *in situ*, permite estudiar procesos específicos en los ecosistemas acuáticos, incluyendo la estructura de las comunidades y los procesos biogeoquímicos que engloban el ciclo de N. Además, la combinación de estas técnicas con otros métodos biológicos, como por ejemplo los que se basan en estudios moleculares, ofrecen una excelente alternativa para explorar en más detalle el funcionamiento de estos ecosistemas desde una perspectiva ecológica.

B. DISSERTATION OBJETIVES

"The scientist is not the person who gives the right answers, he is one who ask the right questions"

Claude Lévi-Strauss

The overarching goal of this thesis was to explore the structure and the functioning of inland water ecosystems by using stable isotope approaches (SIA). Since nitrogen processes are critical for aquatic ecosystems functioning, we, mostly, focused on the use of ^{15}N to assess both food web structure and biogeochemical processes involved the aquatic N cycle. This is a dissertation framed on system ecology and we spotlighted in the perspective of whole-ecosystem approach because we consider this holistic outlook as the best way to assess functional aspects through the interactions among all different ecosystem components (biotics and abiotics). The main objectives were addressed in five separately, but complementary, chapters:

In the **first chapter**, the first objective was to evaluate the factual use of ^{13}C and ^{15}N natural abundances and community-wide metrics in a whole ecosystem perspective to assess quantitatively differences on lake food web structure. In this chapter we quantified lake´s structure differences by using a comparative approach of 10 environmental contrasted lakes and check the role of abiotic environmental attributes as main drivers defining the structure of food webs. To this end, we combined the use of trophic links and chain descriptors with^{13}C and ^{15}N based community-wide metrics (Layman metrics).

The main goal of the **second chapter** was to assess the major issues arising from the use of the stable isotopes (^{15}N and ^{13}C) addition as tracers in whole-scale ecosystem studies. To this end, we made a conscientious compilation of those experiments using SI tracer additions at entire-ecosystem scale performed until today in freshwaters, which were reviewed in order to bring light all the potential applications behind this approach solving the main paradigms on aquatic ecology.

The **second and third chapters** were dedicated to assess N dynamics on inland waters through whole ecosystem field-based $(^{15}NH_4)_2SO_4$ additions. Specifically, chapter 3 explored N cycling and retention in a small 3er order stream influenced by agriculture; whereas the chapter 4 assessed the N transformations and uptake rates by the main biotic compartments in a oligotrohic lake.

The main goal of the **fifth chapter** was to determinate the potencial N enzymatic pathways associated with the N cycle (i.e., nitrification, denitrification, DNRA and anammox) in the aquatic systems where ^{15}N tracer additions were performed. To this end, genes abundances by q-PCR analyses were measured and the occurrences of main N enzymatic pathways were determined. Finally, by means of a multi approach perspective which combined biogeochemical and stable isotope analyses with molecular (abundance of functional gene) methodologies, N dynamics in both aquatic systems is evaluated.

C. CHAPTERS

CHAPTER 1: Main environmental attributes defining patterns in the food web structure of lakes: stable isotope-based community metrics

CHAPTER 2: Successes, limitations and challenges facing the experimental addition of ^{13}C and ^{15}N as tracers in freshwaters at ecosystem scale

CHAPTER 3: Testing nitrogen dynamics in a stream influenced by agriculture: ^{15}N tracer addition approach

CHAPTER 4: Nitrogen dynamics in an oligotrophic lake through and ecosystem-scale isotope tracer experiment

CHAPTER 5: Abundance and distribution of functional genes encoding key enzymes in the N cycle of freshwaters

CHAPTER 1

Main environmental attributes defining patterns in the food web structure of lakes: stable isotope-based community metrics

"The first law of ecology is that everything is related to everything else"

Barry Commoner

1.1 SUMMARY

In lakes, biotic and abiotic environmental variables are expected to directly and indirectly alter the food-web dynamics and community structure. However, explanations behind the mechanisms often continue to be a fundamental ecological issue. Stable isotope-based community-wide metrics, in combination with trophic links and chain length measures, may potentially increase the ability to provide a compelling description of the relationships of these attributes with ecosystem structure. In this study we estimated trophic structure patterns of 10 dissimilar lakes, and we assessed main environmental attributes defining lake structure. To this end, we first collected all possible data related to the ecosystem structure of the studied lakes from the literature, including $\delta^{13}C$-$\delta^{15}N$ values. Then, we calculated main trophic link descriptors and Layman community metrics. For that, we used standardized data (by calculating Z-scores), which allows us to compare Layman metrics, minimizing the trouble associated to the gross SI magnitudes. Our results suggested that lake communities of greater diversity contain species that are more packed but less connected and tightly coupled in functional groups, which agrees with the interpretation of lake structure as highly interconnected food webs, rather than as linear food chains. Furthermore, several lines of evidence indicated that depth, elevation, latitude, and the chlorophyll-*a* content are the most important abiotic environmental drivers defining lake community structure.

Keywords: stable isotope analysis, ecosystem-structure, community-metrics, lakes, link properties, environmental attributes

1.2 INTRODUCTION

Understanding the mechanisms underlying ecological communities and their dynamics is a fundamental goal of ecology (Mutshinda and O'Hara, 2011). The analysis of trophic structure has been used to describe communities and species functional relationships on freshwater ecosystems (Cardinale, 2011; Thompson et al., 2012; Abrantes et al., 2014). The relative importance of biotic and abiotic factors in the regulation of these ecological systems has been debated throughout the last decades (McIntosh, 1985; Jackson et al., 2001). In lakes, food-web complexity has been related with abiotic environmental settings, such as watershed characteristics (including ecosystem size and chemical inputs) and lake-internal processes (Perkins et al., 2010; Jeppesen et al., 2010, 2012; Cooper and Wissel, 2012). Today, a major challenge for ecologists is to predict how ecosystems change with environmental conditions. To further our understanding of these responses, it is important to be able to disentangle the specific effects of each attribute in the ecology of the community, because explanations behind many of these mechanisms continue to be a fundamental ecological issue (Duffy et al., 2005; Macfadyen et al., 2009; Cooper and Wissel 2012).

Several trophic descriptors have been developed to extract ecologically meaningful information from the trophic space, characterizing connections among species (Bersier et al., 2002; Schmidt et al., 2007). These approaches have been designed to characterize feeding relationships. The structure of the food web is described by means of the trophic links, giving basic information on food chain length, connectance and the mass and energy flux through the trophic levels (Pimm, 1982, 2002; Post et al., 2002; Melian et al., 2004; Anderson and Sukhdeo, 2011; Perkins et al., 2013). While these descriptors

are mainly based in the total number of species, stable isotope-based metrics provide a integrated scope of dietary pathways, quantifying numerous aspects of community structure and food web ecology (i.e. trophic diversity, population and ecological niche, species packing and food web linkages; Layman et al., 2007; Jackson et al., 2011, 2012; Bearhop et al., 2004, Newsome et al., 2007; Schmidt et al., 2007).

The most accepted stable isotope-based food-web metrics on a community level are those proposed by Layman et al. (2007). These community metrics move ahead of qualitative description of the position of a species on the $\delta^{15}N$-$\delta^{13}C$ in the bi-plot, providing means for basic comparisons. There are four metrics which are suggested to summarize different aspects of trophic diversity: $\delta^{15}N$ range (NR), $\delta^{13}C$ range (CR), total area of the convex hull encompassing the data points (TA), and the average Euclidian distance to the centroid, mean distance to centroid (CD). Two metrics describe the relative spacing of species in the isotope bi-plot and are suggested to be an indicator of trophic redundancy: mean nearest neighbor distance (MNND), and standard deviation of the nearest neighbor distance (SDNND). The recent availability of these quantitative descriptors should increase the ability to provide compelling patterns of lake food web structure and the main control mechanisms. The sense of these community metrics is to compare different scenarios (space or time), but results from the metric scores themselves cannot be directly compared among different ecosystems without accounting for isotope variation in the resource or prey items (Syväranta et al., 2013). While such variation might be small in some cases, such as some terrestrial systems, lake ecosystems can often show considerable differences (e.g. population utilizing both littoral and pelagic prey sources in one lake can generate smaller isotopic signatures than a population of conspecifics in

another lake, even though feeding on exactly the same prey, simply because the variation in prey $\delta^{15}N$-$\delta^{13}C$ differs between the lakes, Syväranta et al., 2013).

The comparison of food web structures among different lakes has been based on simulated data (e.g. Hoeinghaus and Zeug, 2008; Brind`Amour and Dubois, 2013), rather than directly observed $\delta^{13}C$-$\delta^{15}N$ data (e.g. Cooper and Wissel, 2012; Abrantes et al., 2013). The main explanation for this absence lies on the weakness of these metrics to compare food webs when $\delta^{15}N$ and $\delta^{13}C$ of sources are not of the same magnitude, needing a proper data-standardization that has not been fully explored yet (Layman and Post, 2008; Jackson et al., 2011). Currently, different techniques are available to standardize stable isotope based metric scores (e.g. calculating Z scores or transforming δ-space from $\delta^{13}C$-$\delta^{15}N$ data into p-values calculated from source proportions, Moreno et al., 2006; Newsome et al., 2007; Olsson et al., 2009). Combining trophic positions and source p-values with Layman metrics scores provides an interesting alternative, but would be limited to studies with clearly defined sources, which is not the case of many worldwide lakes studies (Newsome et al., 2007). The use of Z-scores (based on a log-transformation to and data normalization, Ricklefs and Travis, 1980), promises great benefits to minimize the uncertainty associated with variability in isotope ratios of prey items and basal sources, but it has not been fully explored yet by aquatic ecologists.

The debate over the abiotic factors regulating trophic structure and interactions has been ongoing for many years. In lakes, Layman´s community metrics have demonstrated that food web structure of saline lakes of northern

Great Plains (Canada) were mainly influenced by salinity (Cooper and Wissel, 2012). Based on link and chain length properties, Vander Zanden and Fetzer (2007) found a positive relationship between ecosystem size and food-chain length of lakes at a global scale. Valdeboncoeur et al. (2003) results indicated that nutrient content and productivity were shown to influence on benthic/pelagic ratios and trophic interactions among lakes in a phosphorous gradient. Recently, two meta-analysis found that latitude, temperature and ecosystem size were the environmental drivers defining food web structure in lake ecosystems, increasing the relative richness of omnivors with decreasing latitude (Gonzalez-Bergonzoni et al., 2012) and increasing the food chain length with the ecosystem size (Takimoto and Post, 2013). Some of these studies focus only on specific populations or feeding status, rather than the whole lake ecosystem. Others explore the effects of abiotic features on lakes located in similar environmental scenarios, minimizing the global scale of the results. The relationships between ecosystem size and nutrient loads on lake structure have been described, but there is still a lack of knowledge of the potential effects by other environmental attributes, such as latitude, altitude, depth or pH in a lake. Moreover, any of these studies have taken advantage of using both approaches, SI community metrics and links and chain length descriptors, which could potentially give a broader insight.

In this study, using links properties and stable isotope-based community metrics of 10 lakes located in dissimilar environmental scenarios, we estimated the trophic structure patterns. Our main goals were, first to assess comparatively the capability of both, trophic link indicators and stable-isotope community metrics, to describe and to compare lake food-web structure; and second, to identify those key environmental variables

influencing lake trophic structure. Ultimately, we also intended to discuss the scope of isotopic metrics to define lake trophic structure using observed data from natural ecosystems, rather than simulated data.

1.3 MATERIAL AND METHODS

Study sites and data collection

The study was undertaken in ten lakes with contrasting environmental settings for which $\delta^{15}N$ and $\delta^{13}C$ stable isotope data on consumers were available (Fig. 1.1). Two contrasted European lakes: Laguna Cueva Morenilla (Spain) and Loch Ness (Scotland); three lakes located in United States: Cascade, Tahoe and Superior lakes, the first two located in the west, while the last one was sited far to the north east of them; two lakes in Canada: Lenore and Fishing lakes; one lake ecosystem located in the south hemisphere, the Moreno Lake (Patagonia, Argentina); and one African lake, the Kyoga from Uganda, a large lake with two distant ecosystems sampled, Lyingo and Bukungu sites. Environmental and isotopic data were obtained from Keough et al. (1996), Grey et al. (2002), Vande Zanden et al. (2003), Mbabazi et al. (2009), Cooper and Wissel (2012) and Arcagnia et al. (2013), and are summarized in Table 1.1. Trophic Status Index (TSI) was calculated as described by Carlson (1977) and trophic status of each lake was defined according to Ryding and Rast (1992).

All sites and methods for environmental variable determinations, sampling species and stable isotope analyses are described in detail in every study, with the exception of Cueva Morenilla, which we sampled in summer 2010 (July, 19-23th) coinciding with the highest primary productivity period.

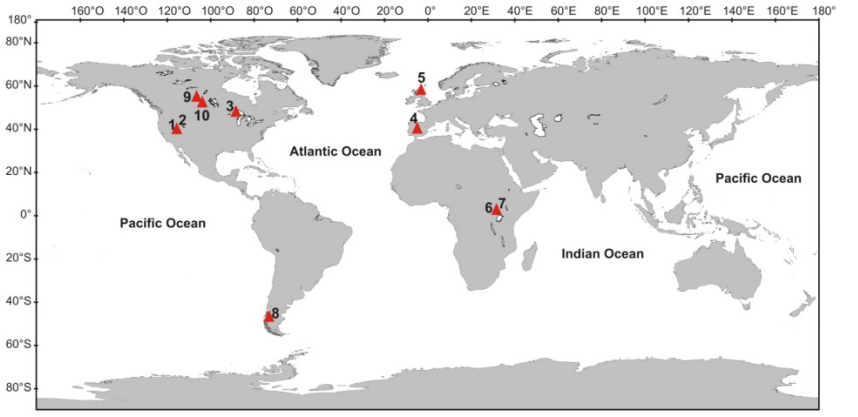

Fig. 1.1: World map of study sites locations. 1: Cascade Lake (Nevada, USA), 2: Tahoe Lake (Nevada, USA), 3: Superior Lake (Wisconsin, USA), 4: Laguna Cueva Morenilla (Spain), 5: Lochness (Scottland), 6: Kyoga Lake (Bukungu shore; Uganda), 7: Kyoga Lake (Lyingo shore; Uganda), 8: Lago Moreno (Argentina), 9: Lenore Lake (Canada), 10: Fishing Lake (Canada).

This lake belongs to a chain of 18 barrage tufa lakes that encompasses the main section of a protected environmental area (Ruidera Lakes Natural Park). This small, karstic, shallow and monomictic Lake is located downstream of the lake complex which is fed by surface water (mainly) and groundwater, and surrounded by a fringe of helophytes encompassed largely by *Cladium mariscus* plants. Submerged plants (mostly charophytes) that form lake wide meadows, were absent during our study period because of strong water discharge which also resulted in a continuous mixing. Zooplankton (composed mainly by rotifers and some cladocerans and copepods), was sampled in a central point by scraping a Nytal filter (>125 μm) after filtering 500 L of lake water. Invertebrates *(Branchiura sowerby, Stictochironomus sp.* and *Caenis sp.*) were obtained by sieving hand-shoveled sediments from the first 5 cm from the surface and by scraping aquatic vegetation, and after preserved in formol (4%). Because individuals from each taxa had no sufficient biomass to be analyzed separately, all invertebrates were gathered

in a composite sample. Crayfishes (*Procambarus clarkii*) and four nonnative fish species (*Cyprinus carpio, Esox lucius, Lepomis gibbosus* and *Gambusia holbrookii)* were captured using nets and traps of different sizes which were submerged in the lake during 24-48 hours. In the laboratory, specimens were measured and weighted, lipids were removed with clorophorm:methanol (2:1) and muscle tissues extracted, dried up at 110ºC, pulverized with a mortar and stored dry until SI were analyzed (Pinnegar and Polunin, 1999; Ventura and Jeppensen, 2009). *Gambusia holbrooki* specimens were too small and, consequently, head and tail were removed and all that remainined was used for SI analyses. For $\delta^{13}C$ and $\delta^{15}N$ content, 1 mg of dried samples were pressed into ultra-pure tin capsules and analyzed by EA-IRMS in the Stable Isotope Laboratories of the University of Arizona and Georgia University.

Results are reported as parts per thousand (‰) differences from a corresponding standard (PeeDee Belemnite for $^{13}C/^{12}C$; nitrogen air for $^{15}N/^{14}N$) with an analytical precision of 0.02 and Standard deviations of $\delta^{13}C$ and $\delta^{15}N$ replicate analyses of 0.14‰ and 0.13‰, respectively. A portable Crison MM-40 multimeter (Crison Instruments; Alella, Spain) was used to measure water conductivity and pH; total nitrogen and total phosphorus contents in the lake water were measured colorimetrically with a Seal-3 QuAAtro AQ2 autoanalyzer (Seal Analytical Ltd., Segesworth, UK) following APHA (2005).

Community metrics assessment

Isotope data from primary producers were lacking in some lakes and, additionally, their isotope ranges seemed to change dramatically between seasons and lakes (Doi et al., 2004), and we decided to use only consumers (primary and secondary) allowing a better comparison of trophic structure among lakes. In all lakes, primary consumers were simplified as zooplankton and invertebrates, while fish and a few crayfish species (*Procambarus clarkii* and *Samastacus spinifrons*) were mainly referred to as secondary consumers. Some of the study sites shared fish species (e.g. Lyingo and Bukunga), but most of them had their specific fish assemblages.

Links trophic indicators: Links properties were based on the total number of species (S) and trophic links (L) and expressed as link density (LD=L/S) and Connectance ($C=L/S^2$) (Warren, 1994). In the L-S relationship, we represented the lower limit (given by L = S - 1) as the minimum number of links required to ensure that no part of a web is disconnected, and the upper limit (L= [S(S - 1)]/2) (Briand, 1983; Auerbach, 1984). Chain properties were estimated as maximum (MAX_{cl}) and mean (M_{cl}) chain lengths. Feeding relationships within species on a lake were determined using averaged $\delta^{15}N$ and $\delta^{13}C$ values, instead of gut-content analyses. To estimate proportional contributions of sources (primary consumers: zooplankton and invertebrates) to the mixtures (upper consumers) and to calculate links, isotope data were incorporated into the Bayesian mixing model SISUS (Stable Isotope Sourcing Using Sampling, http://statacumen.com/sisus/, Erhardt et al., 2014) computed in the 'R' statistical software version 3.1.2 (R Development Core Team). In the mixing analyses, we only took into account the sources that

represented a feeding relationship higher than 10 % of the total. MAX_{cl} and M_{cl} were calculated following Post (2002):

$$TL_i = \lambda + (\delta^{15}N_i - \delta^{15}N_{PC}) / F \quad \text{(Eq.1.1)}$$

where TL_i is the average trophic level of the species i, $\delta^{15}N_i$ is the average content, $\delta^{15}N_{PC}$ is the average content of primary consumers (in our case this value is an average of the zooplakton and invertebrates), λ is the trophic level of consumers estimating the base of the food web (2) and F is the per trophic level fractionation of $\delta^{15}N$ (we used the fractionation value of 2.54, Vanderklift and Ponsard 2003).

Layman community-wide metrics: Isotope data used to calculate Layman metrics was previously standardized. First, isotopic data were log-transformed to equalize variances of $\delta^{15}N$ and $\delta^{13}C$ measurements and make them equivalent, and then standardized by calculating Z values:

$$Z = (x-X) / s \quad \text{(Eq.1.2)}$$

where x is the isotopic values of each species, X is the mean value of the isotope (all consumers) and s is its standard deviation (Moreno et al., 2006). Layman Stable isotope community metrics (NR, CR, CD, MNND, TA and SDNND) were calculated using the package SIAR (Parnell et al., 2008, 2010) computed in the 'R' statistical software. The methodology for calculating the population metrics is available in Jackson et al. (2011, 2012). Although some authors have suggested the use of Bayesian standard ellipse areas (SEA_c), we decided to use convex hulls areas because SEA_c has been proposed mainly for the study of ecological niches and population whereas TA is based on community assessment (Jackson et al., 2011; Abrantes et al., 2013; Syvaranta et al., 2013).

Statistical analyses

Principal components analysis (PCA) with the environmental data of each lake (mean depth, total nitrogen, total phosphorus, chlorophyll-*a* content, conductivity and pH) was used to infer the similarity among lakes. Tree diagrams cluster was performed to work out lake assemblages based on trophic structure likeness. Non-parametric Spearman correlations were used to test relationships between links and community metrics and environmental attributes. All these statistical analyses were performed with STATISTICA software v.6 for Windows (StatSoft 2001).

Multivariate Regression Tree analyses (MRT) were calculated in the package 'mvpart' (Therneau and Atkinson, 2009) within the 'R' statistical software. This statistical technique is a method to determine clusters defined by a set of environmental values, and has been used to explore, describe, and predict relationships between multispecies data and environmental characteristics (De'Ath, 2002; Larsen and Speckman, 2004). However, in this study we used this approach to establish associations between food web structure of lakes (through qualitative and quantitative metrics; response variables) and environmental features (predictor variables), which means that each cluster represents lake trophic assemblages that have been defined by environmental variables (node). This analysis makes no assumptions about the form of relationships (e.g. unimodal or linear), and is applicable for complex ecological data with imbalance, non-linear relationships between variables and high-order interactions (De'Ath and Fabricius, 2000). No standardized SI data were used to assess the effects of environmental variables on lake food web structure.

1.4 RESULTS

Our 10 study lakes showed a broad range of environmental settings, with 2 sub-alpine (~2000 m above sea level), 6 of moderate altitude (900-500 m), and 2 lowlands (< 200 m) lakes, with mean depths ranged from 2 to 313 m and large differences in trophic status, nutrient concentrations, water chemistry parameters and appearance of exotic species (Table 1.1).

Regardless of this disparity, results from the PCA indicated closer water-quality properties among Loch Ness, Moreno, Cascade and Tahoe, which can be classified as oligotrophic deeper lakes (>30 m) with lower nutrient and water chemistry values (Fig. 1.2). In contrast, Lyingo and Bukungu are classified as eutrophic shallow lakes (<6 m), while Superior, Fishing, Lenore and Cueva Morenilla, matched together, have values close to mesotrophic and low mean depths. The first (PCA1) and second (PCA2) axes were both significant and explained 46.3 % and 33 % of the observed variance in environmental variables, respectively.

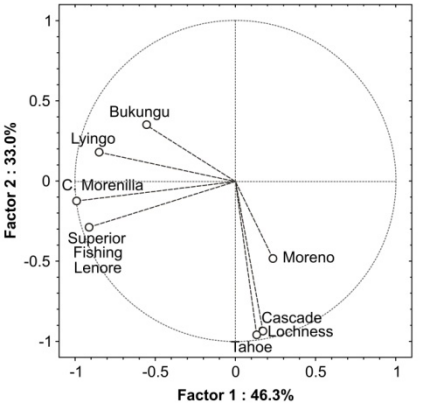

Fig. 1.2: Principal components analysis of the study sites and their abiotic characteristics (pH, conductivity, chl-*a*, nitrate, total phosphorous and mean depth).

Table 1.1: Geographic, limnological and water chemistry characteristics of the 10 studied sites: Cascade, Cueva Morenilla, Lochness, Bukungu, Lyingo, Tahoe, Superior, Moreno, Lenore and Fishing: "% Native" refers to the weight of exotic species in each lake and is also calculated as [(native –non-native / native + non-native) *100]. References: 1: Vander Zanden et al. (2003), 2: This study, 3: Grey et al. (2002), 4: Mbabazi et al. (2009), 5: Keough et al. (1996), 6: Arcagnia et al. (2013), 7: Cooper and Wissel (2012).

Variable	Cascade	Cueva Morenilla	Lochness	Bukungu	Lyingo	Tahoe	Superior	Moreno	Lenore	Fishing
Location	Nevada (USA)	Ciudad Real (Spain)	Highlands (Scotland)	Busoga (Uganda)	Busoga (Uganda)	Nevada (USA)	Wisconsin (USA)	Rio Negro (Argentina)	Saskatchewan (Cánada)	Saskatchewan (Cánada)
Latitude (°)	40	40	55	0	0	40	45	41	52	52
Elevation (m)	1998	760	16	914	914	1998	180	766	537	529
Depth (m)	30	8	132	6	3	313	2	67	9	12
Secchi disc (m)	>15	<15	>15	<15	<15	>15	<15	>15	<15	<15
pH	8.75	7.8	6.5	7.8	8.4	7.5	7.2	7.16	8.5	8.4
Conductivity (µs/cm)	10	750	33	121	268	92	97	37	1700	3124
Chl-*a* (µg /l)	0.46	1.30	1.50	26.23	31.20	0.60	0.40	1.45	15.00	14.00
N-N0$_3$ (mg/L)	1.1	9.5	0.1	8.00	8.00	0.013	0.35	2	0.002	0.00045
TP (µg /l)	3.79	300.00	10.00	300.00	300.00	6.30	3.00	4.02	9.00	37.00
Average TSI	23	60	36	75	75	28	21	29	46	56
Trophic Status	Oligo	Meso	Oligo	Eutro	Eutro	Oligo	Oligo	Oligo	Meso	Meso
% Native spp	67	0	100	40	40	33	25	33	100	80
References	1	2	3	4	4	1	5	6	7	7

Food web descriptors

Links and chain length properties- Study lakes hold between 4 to 10 consumers, structured on maximum chain lengths (MAX_{cl}) ranging from 3 to 4 trophic levels, typical values of lentic systems (Table 1.2; Lamper and sommer, 1997). Fig. 1.3a showed a composition of the food webs based on the relationship between trophic links and the number of species. Data shows that lakes are close to the lower limits of L-S relationship. As species richness (S) increased, the number of links (L) is enhanced while the connectance values (C) declined (Spearman rank order correlation $R>0.76$, 0.78; $p<0.05$). Lenore and Superior were plotted closer to the upper limit, having the highest connectance values (>0.3), while Tahoe and Fishing are graphed below to the lower limit line, showing lesser connectance values (<0.16) (Fig 1.3a). The rest of the lakes are closer, but above the limit line, with intermediate connectance values (0.3 to 0.13). The averaged trophic levels (M_{cl}) were positively correlated to the total number of links (L) (Spearman rank order correlation $R>0.73$; $p<0.05$).

Cluster analysis of food web properties suggested two major lake-assemblages: one node placed together Lyingo and Bukungu with Moreno and Superior lakes, while the other node fitted together the clusters Cascade-Cueva Morenilla and Lochness-Fishing-Lenore with Tahoe lake (Fig. 1.3b).

Table 1.2: Links properties and Isotope-based community metrics defining the trophic structure of the study sites. Web size (S) means number of secondary consumers, and in the text it is also referred to as species richness. Link Density was estimated as LD=L/S, and Connectance means $C=L/S^2$. Chain length is calculated as the maximum (MAX_{cl}) and mean trophic levels length (M_c). SI metrics were calculated with gross and standardized $\delta^{13}C$ and $\delta^{15}N$ data, calculated as Z scores.

	Cascade	Cueva Morenilla	Lochness	Bukungu	Lyingo	Tahoe	Superior	Moreno	Lenore	Fishing
Links and chain properties										
Web size (S)	6	5	4	10	10	9	6	9	4	5
Links (L)	7	7	5	15	13	6	12	11	6	4
Density (LD)	1.2	1.4	1.3	1.5	1.3	0.7	2.0	1.2	1.5	0.8
Connectance (C)	0.19	0.28	0.31	0.15	0.13	0.07	0.33	0.14	0.38	0.16
MAXcl	3.47	3.91	3.74	3.87	3.66	3.69	3.85	3.64	3.05	3.17
Mcl	2.96	3.29	3.01	3.35	3.26	3.16	3.41	3.04	2.78	2.91
Stable Isotope (gross-data)										
NR	5.62	8.75	7.50	7.49	5.65	6.09	7.30	6.50	5.33	5.77
CR	14.47	10.06	8.70	5.18	7.78	11.62	4.40	13.00	5.67	4.92
TA	46.88	34.19	36.90	21.80	21.90	47.80	7.56	46.11	22.09	15.80
CD	3.65	3.29	3.56	2.08	1.96	3.65	2.22	3.13	2.70	2.06
MNND	2.78	2.85	2.78	1.12	1.26	1.80	1.21	1.64	1.85	1.36
SDNND	1.59	1.33	0.28	1.01	1.30	1.11	1.05	1.26	0.63	0.97
Stable Isotope (Standardized)										
NR	3.02	3.23	2.94	3.65	3.72	3.13	2.97	3.39	2.63	3.01
CR	3.44	3.24	3.07	2.35	3.62	3.27	3.63	4.72	2.66	3.32
TA	6.12	3.68	5.13	6.27	6.72	7.28	2.20	8.46	5.05	5.49
CD	1.20	1.11	1.32	1.09	1.01	1.23	1.23	1.30	1.30	1.17
MNND	0.94	0.93	1.05	0.48	0.68	0.68	0.76	0.65	0.84	0.81
SDNND	0.48	0.55	0.29	0.83	0.84	0.54	0.61	0.52	0.33	0.59

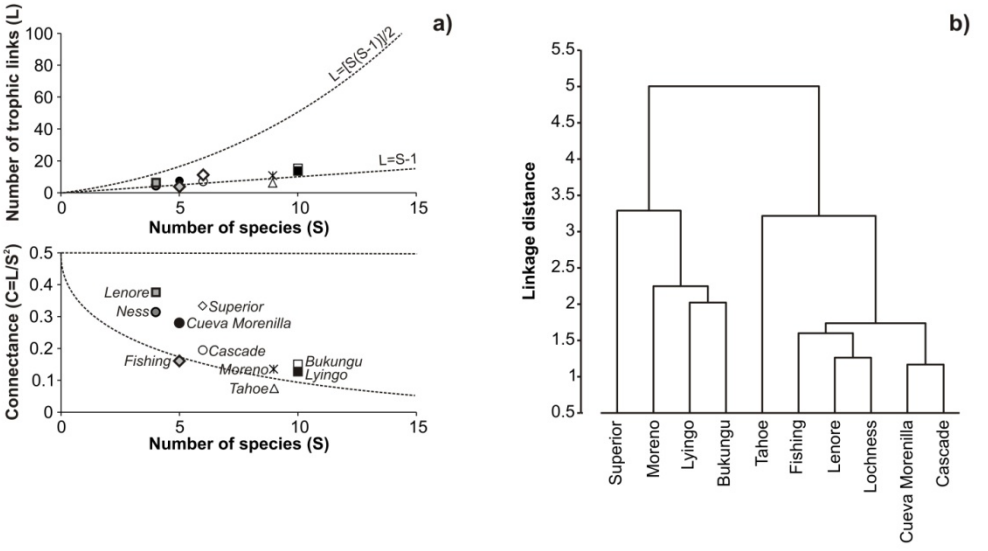

Fig. 1.3: (a) Number of species *vs.* the number of total trophic links and the connectance of each lake. In the upper graph, dotted lines represent the predicted lower and upper limits. In the lower graph, the dotted line traced the tendency line based on the plotted results. (b): Tree diagram cluster grouping the study lakes based on the link and chain descriptors: species richness, number of links, link density, connectance and maximum and mean chain lengths.

Layman community metrics- $\delta^{15}N$ and $\delta^{13}C$ gross signatures from primary and secondary consumers of each lake food web were plotted in bi-plots shown in Fig. 1.4, and metrics (gross and standardized) results from SIBER are summarized in Table 1.2. Fig. 1.4 showed how the consumers and their potential sources (primary sources) are distributed, and the $\delta^{15}N$ and $\delta^{13}C$ ranges (note the magnitude of axis scales).

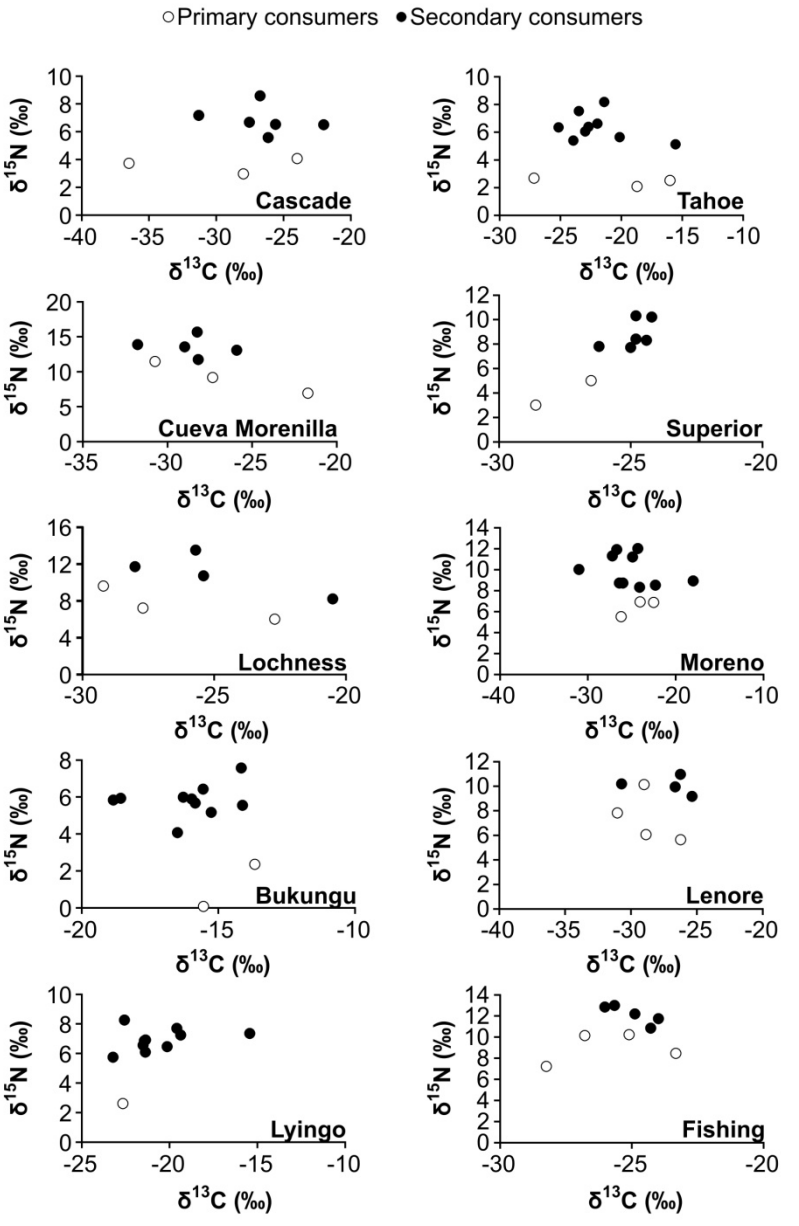

Fig. 1.4: $\delta^{13}C$ and $\delta^{15}N$ mean gross signatures of study primary (white circles) and secondary (black circles) consumers of each lake. Primary consumers refer to the baseline of our food web and included invertebrates and zooplankton. Secondary consumers included crayfish and fish species.

Based on the standardized data, NR exhibited a positive correlation with TA (Spearman rank order correlation R= 0.64; $p<0.05$). An inverse relationship between NR and MNND was found (Spearman rank order correlation R=-0.68; $p<0.05$). We did not find any significant correlation between CR values and the other Layman metrics.

Tahoe and Moreno exhibited the greatest TA (Table 1.2) with more than 70 % of overlap between them (Fig 1.5b). Superior and Cueva Morenilla showed the lowest TA with an overlap of <50 % (Fig 1.5b, Table 1.2). High TA measures might indicate greater occupied isotope niche encompassing the entire food web (consumers). In systems with equal number of species, such as Cascade and Superior lakes, significantly lower TA is associated with more packed communities (Table 1.2). Non-parametric correlations showed that TA tended to increase as species richness (S) and NR rose (Spearman rank order correlation R= 0.70, 0.64; $p<0.05$). The other metric related to convex hulls, mean distance to centroid (CD), exhibited a negative correlation with NR and SDNND (Spearman rank order correlation R= -0.72, -0.85; $p<0.05$).

Results also showed a negative tendency across communities, with decreasing MNND values as species richness (S) and number of links (L) increased and connectance (C) decreased (Spearman rank order correlation R=-0.84, -0.65, 0.64 ; $p<0.05$). NR exhibited the opposite trend (Spearman rank order correlation R= = 0.86, 0.68, -0.78; $p<0.05$).

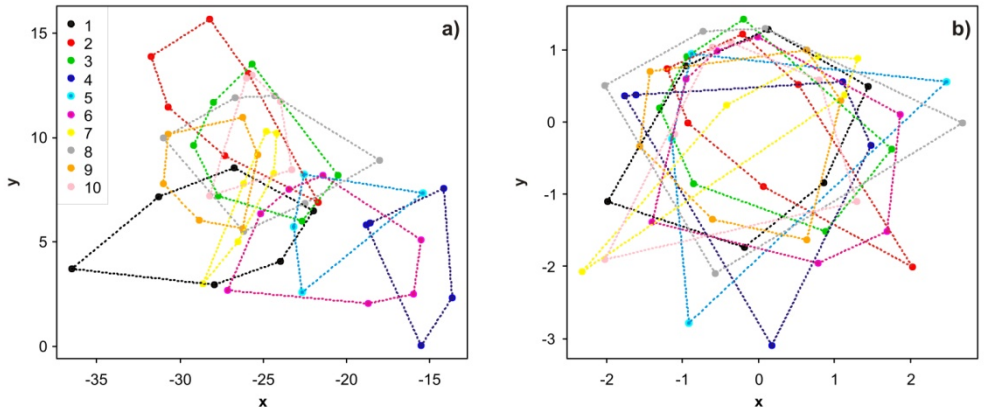

Fig. 1.5: Convex Hulls graphs with the (a) gross and (b) standardized $\delta^{15}N$ and $\delta^{13}C$ signatures of all the consumers. Standardized data means Z scores calculated for all the isotope signatures. Dotted lines endorse the convex hull areas of each lake, corresponding to the area encompassing all of consumers in the $\delta^{15}N$- $\delta^{13}C$ plot. Cascade (1), Cueva Morenilla (2), Lochness (3), Bukungu (4), Lyingo (5), Tahoe (6), Superior (7), Moreno (8), Leonore (9) and Fishing (10).

Environmental factors

Incorporating the six links and chain descriptors (defining the food web structure) and the environmental attributes (characterizing the ecological scenarios of the study lakes) in the Multivariate Regression Tree analyses (MRT), results showed that lakes were first split by their depth values, which account for about 60 % of the variation in the original data set (Fig. 1.6). Latitude and chl-*a* were the next main attributes, accounting for 52 % of the variation. The contributions of the rest of environmental parameters was too small to be represented (< 40 %). These results are in concordance with the relationship found between depth and the number of links (L) and Links density (LD) observed in the food webs (Spearman rank order correlation: R=-

0.65, -0.78; $p<0.05$). We also found that Latitude was negatively correlated to Species richness (S) (Spearman rank order correlation: R =-0.84; $p<0.05$). Lakes with higher depths were then split attending on their TP content and Elevation values, explaining up to 54 % of the variation. Elevation was found to be correlated to species richness (S) (Spearman rank order correlation: R =0.68; $p<0.05$).

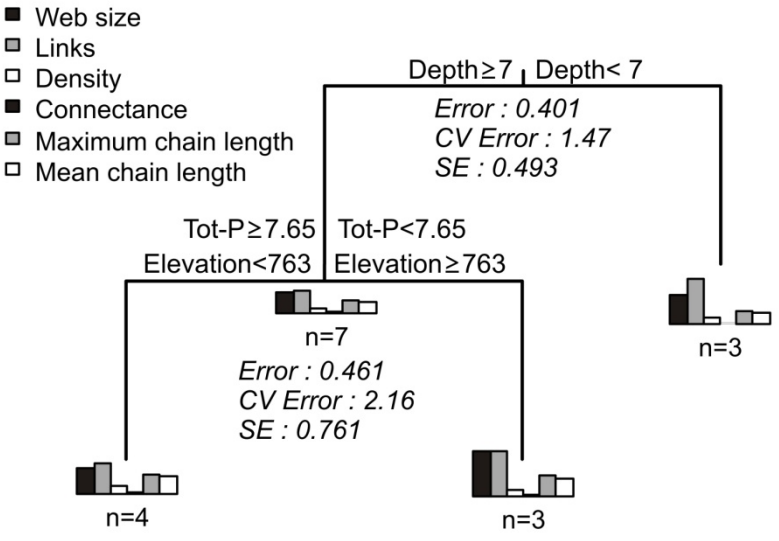

Fig. 1.6: Multivariate Regression Tree analyses (MRT) with the links-chain properties of each food web and the environmental features (latitude, altitude, mean depth, total nitrogen, total phosphorus, Chlorophyll-*a* content, conductivity and pH).

Results from the MRT analyses with the stable isotope-based community metrics, pointed out that elevation was the first attribute splitting Layman metrics, accounting about 55 % of the variation, followed by Latitude with 30 % of variation (Fig. 1.7). Regression analysis found that Elevation and Latitude were correlated with SDNND metric (Spearman rank order correlation: R

=0.66, -0.67; p<0.05). Second branch (less elevation values) split lakes by the nitrate and chl-*a* content and the attributes latitude and depth, account 80 % of the variation each. Regressions showed correlations between chl-*a* and CD (Spearman rank order correlation: R =-0.66; p<0.05), nitrate and SDNND (Spearman rank order correlation: R =0.64; p<0.05) and depth and the metrics CR, TA and CD (Spearman rank order correlation: R =0.65, 0.81, 0.72; p<0.05). Lakes with higher elevation values were assemblage based on the depth and pH values, accounting for 52 % of the total variation and regression analyses found a negative relationship between pH and NR (Spearman rank order correlation: R =-0.73; p<0.05).

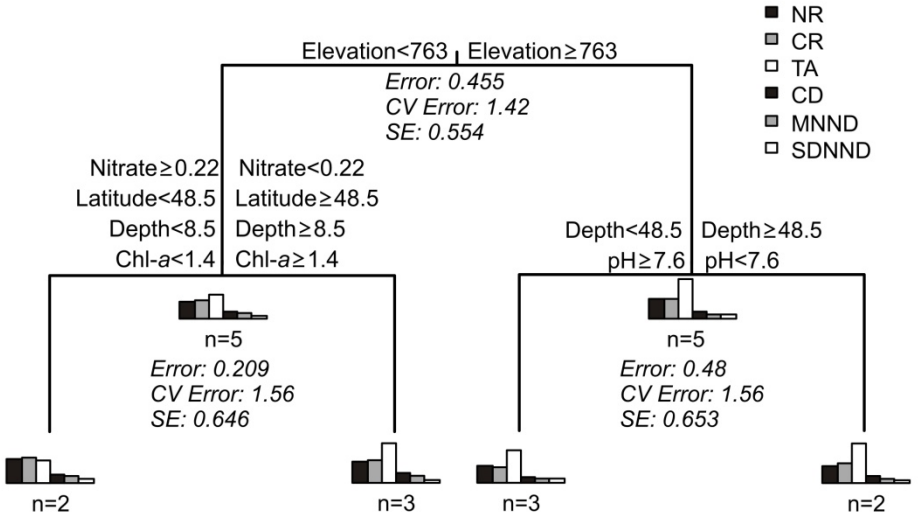

Fig. 1.7: Multivariate Regression Tree analyses (MRT) with the SI Bayesian wide-metrics of each food web and the environmental features (latitude, altitude, mean depth, total nitrogen, total phosphorus, Chlorophyll content, conductivity and pH).

1.5 DISCUSSION

Data standardization allows us to compare not only all the convex hulls, but also the rest of the Layman metrics together, without having troubles with the magnitude of the gross SI isotope signatures. However, Layman scores, by themselves, give poor information comparing lake structure among dissimilar lakes. SIA is an important tool to provide insights of lake´s structures, but they are not a substitute for using more basic descriptors, and the combination of both approaches offer a better data interpretation.

Link properties and stable-isotope based metrics describing lake´s food web structure

Clearly, our results provide evidence that lake food chain length is associated with biodiversity (Perkins, 2013). In food webs, the manner by which the number of trophic links are enhanced in relation to the number of species gives us information about how organisms are structured. Warren (1990) noted that food webs with the trophic links closer to the upper limits corresponded to webs dominated by generalist species, while food webs closer to the lower limits are dominated by specialists (i.e. exploit only very limited proportions of morphological space). Observed webs cannot, by definition, fall below the lower limits line. A feasible explanation when $L < S - 1$ in a community (e.g. Fishing and Tahoe lakes), is that two or more smaller food webs are observed, each with $L > S - 1$. In fact, some of the secondary consumers in these lakes clearly show a preference of some sources over the others (i.e. zoo *vs.* invertebrates) which may indicate two well defined food pathways (i.e. benthic *vs* pelagic). However, we should also assume that food webs are not static

structures (Warren, 1990), especially in aquatic systems, and variation in S (spatially or temporally), will move the web along some particular L-S pattern, and we should be prudent when expecting single linear L-S relationships for data on webs from different ecosystems.

The hypothesis of sub-food webs, or blocks, within a community is also associated with the connectance of species declining with increasing species richness, probably in order to remain as stable food webs (Moore and Hunt 1988; Warren, 1990). The relationship between diversity and stability has fascinated ecologists since many years ago, being the center of scientific debates (e.g. Gardner and Ashby, 1970; Pimm, 1984; Armstrong and McGehee, 1980; Yodzis, 1980). Recent advances indicate that diversity can be expected to increase ecosystem stability (Worm and Duffy, 2003). However, evidences also indicate that this linkage depends on the scale of inquiry, and that diversity is not the driver of this relationship; rather, ecosystem stability depends on the ability for communities to contain functional groups, that are capable of differential response (McCann, 2000). Therefore, communities of greater diversity may contain tightly coupled subunits or functional groups, as connectance, within these compartments, strength would decline (McNaughton, 1978). Accordingly, species richness would primarily reflect diversification rather than species interactions (Ricklefs, 2012). Moreover, nowadays ecologists have replaced the conceptualization of the ecosystem as a linear food chain with the view that food webs are highly interconnected assemblages (Winemiller, 1990; Strong, 1992, Dodds, 2002; Wetzel, 2001). These assumptions could be behind the explanation of the lower connectance values in Moreno, Tahoe, Bukungu and Lyngo lakes found in our results.

The correlation exhibited between connectance values and the mean nearest neighbor distance (MNND) indicated that more connected communities showed less packing. Those results were confirmed through the negative relationship between MNND and species richness and number of links. Therefore, according to the species packing mechanism formulated by Ricklefs and Miles (1994), in lakes, diversity increases clustering density with species being more packed but less connected. Consequently, species found in Bukungu, Moreno, Lyingo and Tahoe lakes are probably more packed due to other factors, as limited resource space, rather than interconnections. In Lenore, Lochness, Cueva Morenilla, Cascade and Superior lakes, low diversity results in lower packed species that are more interconnected. Fishing lake is in the middle, showing low species numbers that are not highly interconnected, neither very packed.

Main environmental attributes defining lake- ecosystem structuring

In lakes, environmental settings are supposed to be one of the main features defining the variability of food-web structures, where species are not randomly feeding across the food webs, but they rather use different structuring strategies (Brind`Amour and Dubois, 2013). Several studies have demonstrated that latitude and temperature (Gonzalez-Bergonzoni et al., 2012), salinity (Cooper and Wissel, 2012), nutrient content and eutrophication (Valdeboncoeur et al., 2003), lake size (Vander Zanden et al., 1999; Takimoto and Post, 2012) and phosphorous content (Carpenter et al., 1992) have a certain degree of influence in defining food web structure of lakes.

Based on both trophic links and food chain properties and SI community metrics, our results indicate that depth, latitude, elevation and Chlorophyll-*a* content are the most important environmental variables influencing community structure of study lakes. At local scales, it is already known that lake basin morphometry determines the relative importance of littoral habitat and the potential of primary producers to whole lake secondary production (Vander Zanden and Vadeboncoeur, 2002). Differences in ratios of littoral surface area to volume and the contribution of different primary producers to consumers may be some of the most plausible reasons explaining the differences in number of links and link density *vs.* depth that we observed in our study. The gradient in food chain length with the latitude, has been also observed in Gonzalez- Bergonzoni et al. (2012) study. Our results also agree with the latitudinal gradient in diversity (i.e. species richness tends to increase from the poles to the tropics) that has been largely reported (Theory of Latitude Diversity Gradient, LDG; Clarke and Gaston, 2006). Elevation gradient is a pattern that has been deeply studied in soil and plant ecology (e.g. Bryant et al., 2008) whereas no much attention has been paid in comparing food webs in aquatic systems. Indirect effects of latitude and elevation may be behind the fundamental role of these parameters defining food web structure of lakes (e.g. solar and thermal radiation or temperature). Control of food web structure by chlorophyll-*a* can be related with the well known "top-down" and "bottom-up" internal regulations that influence the consumers level and how they are structured (Carpenter et al., 1987; Hunter and Price, 1992). Unfortunately we were not able to analyze other controlling factors of food-web structures in our study, such as biotic (e.g. predation, and competition) and abiotic (e.g. osmotic stress, disturbances, landscape) factors, which may also influence food webs.

In conclusion, comparing different lake structures, our results imply that the combination of Layman metrics scores and trophic links and chain descriptors offer a better data interpretation than Layman scores by themselves. Our results provide evidence that lake food chain length is associated with biodiversity. Moreover, we also suggest that, lake communities of greater diversity, in order to remain stable, contain species that are more packed but less connected and tightly coupled in blocks or functional groups. Finally, we found depth, latitude, elevation and chlorophyll-*a* content the most important environmental drivers influencing lake community structure. We postulate that differences in ratios of littoral surface area to volume and contributions of different primary producers, the Theory of Latitude Diversity Gradient and indirect effects of latitude and latitude, and "top-down" and "bottom-up" internal controls are some of the most plausible mechanisms explaining the role of these environmental attributes defining lake structure.

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CHAPTER 2

Successes, limitations and challenges facing the experimental addition of ^{13}C and ^{15}N as tracers in freshwaters at ecosystem scale

2.1 SUMMARY

Deliberate *in situ* ^{13}C and ^{15}N additions in whole freshwater ecosystems have been shown as a powerful tool, contributing to our knowledge of ecosystem processes. ^{13}C and ^{15}N additions to aquatic ecosystems represent valuable advances in the study of aquatic metabolism, basal resources, food web structures and carbon and nitrogen cycling. Currently, this approach can be considered as a powerful complementary tool for studying ecological and biogeochemical processes at whole aquatic ecosystem scale. Despite decades of whole-ecosystem stable isotope additions to aquatic environments, the current scope and future potential on issues related to aquatic ecology and biogeochemistry of this novel technique have not been assessed yet. We intend to fill this gap by providing a comprehensive review addressing the main subjects arising from the use of ^{13}C and ^{15}N as tracers in whole-scale aquatic ecosystem studies. Specifically, we focus on the following questions: (1) Why are SI additions a powerful complementary approach to studying ecological and biogeochemical processes at whole-ecosystem scale? (2) To date, what issues have been resolved with ^{13}C and ^{15}N additions? (3) What are the shortcomings and limitations to their use? (4) What are the current trends and future perspectives of this approach? We identify key research avenues whereby ^{13}C and ^{15}N additions at whole-ecosystem scale would broaden our understanding of aquatic ecology and biogeochemistry. To conclude we will make suggestions for the future development of this approach.

Key words: ^{13}C, ^{15}N, stable isotopes, tracer addition experiments, ecosystem scale, aquatic processes

2.2 INTRODUCTION

The ecosystem scale approach considers a framework for the study of all biotic and abiotic components and processes and its interactions within defined boundaries (see Likens, 1992). Although experimental manipulations of entire ecosystems are faced to several constrains (pseudoreplication, for example), carefully designed experimental manipulation may be considered as one of the most powerful, scientific approach for studying process level questions relative to ecosystems (Likens, 1992). Several studies focused on processes at the ecosystem-level based on nutrient enrichment experiments have contributed to increase our knowledge on aquatic ecosystem functioning; however, this approach cannot shed light on the mechanisms or compartments where nutrient transformations are conducted (Drake et al., 2009). The ecosystem-scale SI addition approach allowed simultaneous examination of transport and processing of C and N and nutrient metabolism as ecosystem processes, which is impossible in traditional bottle or mesocosm experiments (Holmes et al., 2000).

The temporal stability of stable isotopes (SI) and the processes governing isotopic fractionation enable their potential use as biogeochemical tracers, allowing measurement of simultaneous ecological and biogeochemical processes at whole-ecosystem scale (Schimel, 1993; Fry, 2006). The whole-ecosystem isotope enrichment approach provides the only way to examine flows through multiple pools simultaneously while maintaining natural hydrologic and biogeochemical gradients (Tobias et al., 2003). To date, we certainly know the processes and have measured rates of carbon and nitrogen transformation in aquatic ecosystems, including mechanisms and control

variables of nutrient transformations and fates (Peterson, 1999; Mulholland et al., 2000a; Hamilton et al., 2001) but their importance at ecosystem scales under different environmental settings are little known yet. Deliberate *in situ* ^{13}C and ^{15}N additions demonstrated to be a powerful complementary approaches in aquatic biogeochemistry supported by the potential of isotope ecology theory with the focus of nutrient enrichments (Peterson and Fry, 1987; Lee et al., 2011).

In situ ^{13}C and ^{15}N additions at whole-ecosystem scale have provided valuable results in the study of carbon uptake, in the relationships between terrestrial and lake biomes (Cole et al., 2002; Kritzberg et al., 2004; Pace et al., 2004; Carpenter et al., 2005; Tailape et al., 2008), in the uptake, turnover, and retention processes of nitrogen in streams (Tank et al., 2000a; Merriam et al., 2002; Hall et al., 2009b), lakes (Hadwen and Bunn, 2005) and estuaries and saltmarshes (Hughes et al., 2000; Gribsholt et al., 2005), and in the trophic dynamics and food web structure of streams and saltmarshes systems (Raikow and Hamilton, 2001; Hamilton et al., 2004; Galván et al., 2012). Much of our current knowledge (theories and paradigms) in freshwater biogeochemistry has been established after decades of whole-ecosystem SI experimental additions. However, this success of most ^{13}C and ^{15}N addition experiments has not been discussed from a holistic perspective yet. Therefore, we must analyze the current scope and future potential of this approach to study issues related to aquatic ecology and biogeochemistry. Here, we intend to fill this gap by addressing the main subjects arising from the use of ^{13}C and ^{15}N as tracers in whole-scale aquatic ecosystem studies. Specifically, we focus on the following questions: 1) Why are SI additions a powerful complementary approach to studying ecological and biogeochemical processes at whole-ecosystem scale? 2) To date, what issues have been resolved with ^{13}C and ^{15}N additions? 3)

What are the shortcomings and limitations to their use? 4) What are the current trends and future perspectives of this approach? To answer these questions, we have reviewed 78 scientific publications reporting *in situ* $^{13}C/^{15}N$ additions in several aquatic environments and their contributions in terms of carbon and nitrogen cycles have been carefully considered.

2.3 ADVANTAGES OF SI ADDITIONS AS A COMPLEMENTARY APPROACH IN ECOSYSTEM SCALE STUDIES

The use of radioisotopes formed the basis of conceptual and methodological issues in the study of several freshwaters (e.g. the use of ^{32}P (and ^{33}P) tracer in stream ecology; Payn et al., 2005). In food-web ecology, the use of stable isotope as tracers at ecosystem scale adds a functional dimension (rate of transfer) to the structural response (numbers and species) commonly assayed in ecosystem experiments (Carman and Fry, 2002). Scientific publications reporting whole-ecosystem experiments using ^{13}C and ^{15}N additions in freshwaters have been increased since 1990 (Fig. 2.1). This approach has been used in a wide range of aquatic ecosystems, with more frequent application to lotic over lentic systems (Fig. 2.1).

Lake metabolism is an obvious example of the benefits of employing stable isotope tracer addition assessing the net heterotrophy of lakes (Cole et al., 2000), instead methods based on dissolved oxygen monitoring (Staehr et al., 2010). Natural abundance of carbon, nitrogen and sulfur stable isotopes can help to distinguish organic matter sources, but often isotopic differences between endogenous and exogenous materials are not distinct (Peterson et al., 1985). Labeling endogenous primary production with ^{13}C addition at

ecosystem scale provided direct measurements of the importance of terrestrial subsides supporting lake food webs (Pace et al., 2004 and 2007).

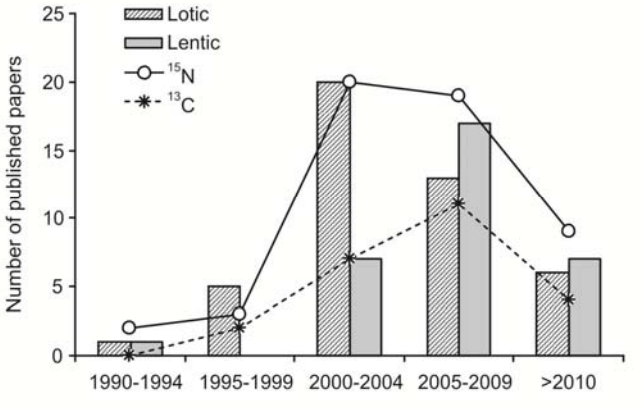

Fig. 2.1: Number of published papers from 1990 to 2013 about *in situ* ^{13}C and ^{15}N tracer addition experiments in aquatic ecosystems. Lentic systems: lakes, intertidal (sand, mud) flats and tidal salt and freshwater marshes; lotic systems: streams (including hyporheic zone) and estuaries.

In food web analysis, multiple food sources have the potential to contribute to the food web. The use of stable isotopes (^{13}C and ^{15}N) natural abundances helped to assess all possible resource combinations to determine a range of possible source contributions (isotope mixing) but results are informative not quantitative (Galvan et al., 2012). Tracer addition increases differences in isotope ratios and when properly designed allow an accurate mass-budget among food web sink and sources, unfortunately, SI addition only trace contemporary production, however, only trace dependence on contemporary production and besides the addition of ^{15}N excludes the use of this SI as a trophic level proxy (Middleburg., 2013). SI additions minimize interpretation problems by amplifying the labeled isotopic signal relative to the variation caused by fractionation (Schimel, 1993). Tracer addition is useful for identifying basal resources supporting secondary production in streams (Raikow and Hamilton, 2001) and in multiple basal resource scenarios such as

tidal saltmarshes (e.g. Galván et al., 2012). Isotopically labeled detritus has been used successfully to trace the flow of C and/or N from detritus to consumers in lakes (Li et al., 2010; Yu et al., 2013).

The main advantage of the whole-ecosystem SI addition approach over the micro/mesocosms approach is obvious, as the former provides *quasi*-actual rate estimates rather than potential rates. Rate estimates by tracer addition of SI are conditioned by multiple constraints related with experimental procedures, including selection of pools to be sampled and sampling frequency. Through a mass-balance of the SI compound added, this technique enables us to estimate whole-ecosystem C and N transformation rates (Gribsholt et al., 2005; Mulholland et al., 2009), including in hydrodynamic complex systems such as estuaries, hyporheic zones and floodplains (Holmes et al., 2000; Hall et al., 2009b; Hubbard et al., 2010; Zarnetske et al., 2011a and 2011b).

2.4 ^{13}C AND ^{15}N TRACER ADDITIONS AT WHOLE-ECOSYSTEM SCALE AND THE MAIN FINDINGS ABOUT AQUATIC BIOGEOCHEMICAL PROCESSES

^{13}C additions: aquatic metabolism, basal resources, and carbon cycling

Deliberate *in situ* ^{13}C enrichments have been performed in aquatic ecosystems through manipulation of the dissolved inorganic carbon pool ($DI^{13}C$; Carpenter et al., 2005), the dissolved organic carbon pool ($DO^{13}C$; Hall et al., 1995) and the organic particulate pool ($PO^{13}C$; Bartels et al., 2012; Table 2.1). Enriched sodium bicarbonate ($NaH^{13}CO_3$ as 98 atom % ^{13}C) has been used extensively in lakes, tidal marshes and estuaries to trace C transfer flows through food

webs (Fig. 2.2; Cole et al., 2002 and 2006; Pace et al., 2004; Carpenter et al. 2005; Kritzberg et al., 2004 and 2006; Pace et al., 2007; Tailape et al., 2008) providing knowledge on lake ecosystem carbon cycles.

This approach is useful to study the aquatic metabolism, particularly in ecosystems where natural SI abundances do not permit separation of autochthonous sources of biological production from allochthonous inputs of organic matter (Hamilton et al., 2004). Whole-lake ^{13}C additions provide strong evidence that terrestrial inputs to lake food webs is widespread across many different kinds of lakes, and that this allochthony is inversely proportional to lake nutrient enrichment (Cole et al., 2002; Pace et al., 2004; Carpenter et al., 2005; Taipale et al., 2008). Results also confirm the hypothesis that, although autochthonous dissolved organic carbon (DOC) is preferentially utilized by bacteria, a large amount of terrestrially derived DOC is respired (not assimilated) by lake bacteria, increasing its importance in oligotrophic systems (Cole et al., 2002 and 2006; Kritzberg et al., 2004 and 2006). In tidal salt marshes and estuarine tidal flats, $NaH^{13}CO_3$ additions demonstrated greater accuracy tracing infauna basal resources compared with natural abundance stable isotope studies. For example, Middleburg et al. (2000) highlighted the central role of microphytobenthos in moderating carbon flow through benthic heterotrophs in coastal ecosystems; Galvan et al. (2012) experiments elucidated the lesser importance of macrophytes (e.g. *Spartina* spp) as food resource for most infaunal species compared with algal resources, including both local (filamentous algae and epiphytic diatoms) and tidally imported (phytoplankton) sources; and Riera et al. (1999) demonstrated that many benthic consumers selectively feed on more nutritious algae.

Table 2.1: Summary of ^{13}C and ^{15}N tracer addition experiments conducted in aquatic ecosystems. Values in brackets in the aquatic ecosystem type column represent the number of SI tracer addition experiments conducted as found in the literature. DIC: Dissolved Inorganic Carbon; DOC: dissolved organic carbon; DIN: dissolved inorganic nitrogen. ([1]) Enrichments using or dextrose compounds are not true $\delta^{13}C$ enrichments but they use the distinctive ^{13}C signature from the natural POM and sediment. (*) Data not found in the literature.

Isotope compound	Aquatic ecosystem type	Isotopic target level (Δδ ‰)	Addition method	Manipulated pool	References
$NaH^{13}CO_3$ (98 Atom % ^{13}C)	Lake (10)	15-40	Daily short pulses (hours) during a couple of weeks	DIC	Cole et al. (2002, 2006); Pace et al. (2004, 2006); Kritzberg et al. (2004, 2006); Carpenter et al. (2005); Solomon et al. (2008); Taipale et al. (2008); Weidel et al. (2008)
	Tidal marsh (4)	*	Surface sediment spraying in instantaneous short pulses (minutes/hours)	DIC	Middelburg et al. (2000); Carman and Fry (2002); Galván et al. (2011); Lee et al. (2011)
$CH_3{}^{13}COONa$ (99 Atom % ^{13}C)	Stream (5)	70-150	Continuous addition (3-4 weeks)	DOC	Hall (1995, 2000); Hall and Meyer (1998); Simon et al. (2003); Parkyn et al.(2005)
Corn (Corn starch or dextrose)	Lake (1)	13-18	Unique addition	POC	Bartels et al. (2012)
	Stream (1)	13-18	Long-term continuous addition (months)	DOC	Wilcox et al. (2005)
$^{13}C_6H_{12}O_6$ (99 Atom % 13C)	Tidal marsh (3)	20-520	Surface sediment spraying in discrete short pulses (hours)	DOC	Van Oevelen et al. (2006a, 2006b); Veuger et al. (2006)

Isotope compound	Aquatic ecosystem type	Isotopic target level (Δδ ‰)	Addition method	Manipulated pool	References
$K/Na^{15}NO_3$ (10-99 Atom % ^{15}N)	Hyporheic zone (2)	10,000	Daily short pulses (hours) during a couple of weeks	DIN (NO_3)	Zarnetske et al. (2011a, 2011b)
	Tidal marsh (7)	650-1,000		DIN (NO_3)	Holmes et al. (2000); Hughes et al. (2000); Tobias et al. (2001, 2003); Galván et al. (2008, 2012); Drake et al. (2009)
	Stream (15)	2,000-10,000	Daily short pulses (2-24 hours) from one day to several weeks	DIN (NO_3)	Peterson et al. (2001); Böhlke et al. (2004); Mulholland et al. (2004, 2006, 2008, 2009); Obrien et al. (2007, 2012); Bernot et al. (2006); Hamilton et al. (2007); Hall et al. (2009a, 2009b); Schiller et al. (2009); Hubbard et al. (2010); Sobota et al. (2012)
	Lake (1)	*	Continuous addition during 10 days	DIN (NO_3)	Epstein et al. (2012)
$^{15}NH_4Cl$ (10-99 Atom % ^{15}N)	Lake (2)	*	Continuous pulses during several weeks	DIN (NH_4)	Kling (1994); Armengol et al. (2012)
	Stream (19)	500-1,100	Continuous pulses during several weeks	DIN (NH_4)	Hershey et al. (1993); Peterson et al. (1997); Hall (1998); Wollheim et al. (1999); Tank et al. (2000a, 2000b); Mulholland et al. (2000a,2000b); Raibow et al. (2001); Sanzone et al. (2001, 2003); Hamilton et al. (2001, 2004); Merrian et al. (2002); Webster et al.(2003); Ashkenas et al. (2004); Parkyn et al.(2005); Morrall et al. (2006); Riis et al. (2012)
	Tidal marsh (2)	*	Unique short injection (hours)	DIN (NH_4)	Carman and Fry (2002); Gribsholt et al. (2009)

Isotope compound	Aquatic ecosystem type	Isotopic target level (Δδ ‰)	Addition method	Manipulated pool	References
$(^{15}NH_4)_2SO_4$ (10-99 Atom % ^{15}N)	Tidal marsh (3)	1,900-4,750	Unique short injection (hours)	DIN (NH_4)	Gribsholt et al. (2005, 2007); Veuger et al. (2007)
	Lake (1)	*	Several short injections every 2 days during 10 days	DIN (NH_4)	Hadwen and Bunn (2005)
^{15}N-organic (*Microcystis*)	Lake (2)	1,800	Unique addition	PON	Li et al. (2010); Yu et al. (2012)

Labile organic compounds such as sodium acetate ($CH_3{}^{13}COONa$ as 99 atom % ^{13}C), glucose ($^{13}C_6H^{12}O_6$ as 99 atom % ^{13}C), and compounds with a distinctive $\delta^{13}C$ stable isotope value, such as corn starch and dextrose, suitable for microbiota uptake, have been added as tracers to study the microbial and invertebrate roles in heterotrophic-based food webs of streams (Hall et al., 1995, 1998; Hall and Meyer, 1998; Simon et al., 2003), lakes (Bartels et al., 2012) and tidal flats (Van Oevelen et al., 2006a, 2006b; Veuger et al., 2006; Table 2.1 and Fig. 2.2).

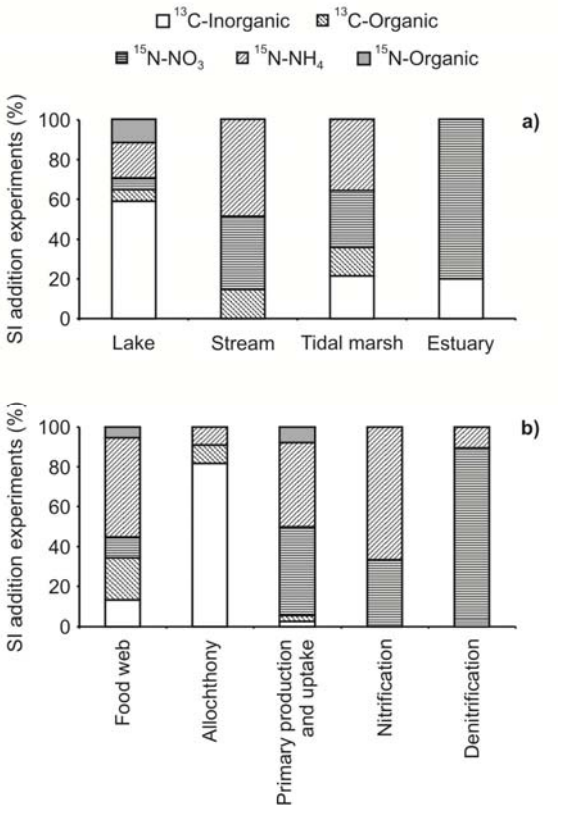

Fig. 2.2: a) Percentage of total ^{13}C and ^{15}N tracer addition experiments by aquatic ecosystem in relation to the isotopic compound used (stream includes hyporheic zone experiments; salt marsh gathers tidal flats, tidal salt and freshwater marshes); b) Main aims of ^{13}C and ^{15}N tracer addition experiments in aquatic systems and the compounds used to achieve these goals.

Whole-ecosystem tracer additions using sodium acetate provide accurate information on the role of bacteria in food webs because this approach integrates several different processes such as DOC uptake by bacteria, and invertebrates feeding on extracellular matter (Hall and Meyer, 1998). In fact, the use of ^{13}C enriched acetate or dextrose through additions in stream reach experiments provided information on interactions between basal resources variability and metazoan food webs in streams: i) heterotrophic biofilms are a significant component of stream food webs (stream invertebrates derive from 10% to 100% of their carbon from bacteria; Hall and Meyer, 1998; Simon et al., 2003; Parkyn et al., 2005, Wilcox et al., 2005), and ii) bacterial carbon is the basic organic source for both biofilm scrapers (Hall et al., 1995; Parkyn et al., 2005) and for predatory invertebrates when bacterial carbon is found in exopolymers (Hall and Meyer, 1998; Hall et al., 2000). Corn starch (a C4 plant) has been used as a POC source taking advantage of its insoluble nature and distinctive $\delta^{13}C$ stable isotope signature. Results of an enclosure experiment using corn starch in a clear-water lake suggested that allochthonous POC is taken by benthic food webs and after further transferred to pelagic systems, thereby highlighting the importance of benthic pathways in pelagic habitats (Bartels et al., 2012). Conversely, additions of enriched organic dissolved compounds such as ^{13}C-glucose show that although a significant fraction of bacterial carbon production is grazed, bacterial carbon is primarily sink of organic carbon in the food web of intertidal flat systems (Van Oevelen et al., 2006a and 2006b). At this site, an experiment adding ^{13}C-glucose to label the bacterial community and subsequently to trace ^{13}C in D-alanine (a peptidoglycan-specific bacterial biomarker, a unique constituent of bacterial wall and the most recalcitrant compound of bacterial remnants) was the first to provide direct *in situ* evidence for the accumulation of peptidoglycan during

reworking and degradation of bacterial biomass in sediments (Veuger et al., 2006).

^{15}N additions: food-web structure and nitrogen cycling

Intentional *in situ* ^{15}N additions to aquatic ecosystems aim to manipulate the dissolved inorganic nitrogen pool ($DI^{15}N$) either via the nitrate ($^{15}NO_3$) and the ammonium pool ($^{15}NH_4$). Manipulation of the nitrogen organic pool has scarcely been undertaken and were based on ^{15}N labelling of algae compartment and subsequently used as enriched detritus (e.g., Li et al., 2010; Yu et al., 2013; Table 2.1 and Fig. 2.2). Potassium/sodium nitrate ($K/Na^{15}NO_3$ as 10-99 atom % ^{15}N), ammonium chloride ($^{15}NH_4Cl$ as 10-99 atom % ^{15}N) and ammonium sulphate ($^{15}NH_4SO_4$ as 10.7 atom % ^{15}N) have been introduced in estuaries, lakes, tidal marshes and streams (Fig. 2.2). Because these ^{15}N compounds can be stored and transferred through trophic pathways, they can be used to assess aquatic food web structures (Hamilton et al., 2004, Galvan et al., 2008) and identify nitrogen uptake rate variability among different habitat types within an ecosystem, as well as nitrogen cycling (Peterson et al., 1997; Hall et al., 1998; Holmes et al., 2000; Sanzone et al., 2001; Gribsholt et al., 2005; Hall et al. 2009a; Fig. 2.2).

The addition of $^{15}NH_4^+$ is an excellent approach to assessing uptake, turnover and retention processes in streams (Peterson et al., 1997; Tank et al., 2000a, 2000b; Hughes et al., 2000; Sanzone et al., 2001; Merriam et al., 2002; Fig. 2.2). $^{15}NH_4$ addition in short pulses has also been used successfully for tracing short-term processes such as nitrification rates and retention dynamics in tidal freshwater wetlands (Gribsholt et al., 2005, 2007, 2009; Fig. 2.2), to assess food-web responses to nutrient inputs (Hadwen and Bunn, 2005; Fig.

2.2) and diel vertical migration patterns of zooplankton in lakes (Armengol et al., 2012). Results from the Lotic Intersite Nitrogen eXperiments (LINX I; www.faculty.biol.vt.edu/webster/linx/), a large scale $^{15}NH_4^+$ addition experiment carried out in 15 headwater streams throughout the US, demonstrated the elevated retentiveness of ammonium by smaller streams (shortest ammonium uptake distances) with uptake distances increasing logarithmically with stream discharge and depth (Mulholland et al., 2000a, Tank et al., 2000b, Peterson et al., 2001, Wollheim et al., 2001, Dodds et al., 2002, Ashkenas et al., 2004, Hamilton et al., 2004, among others). Results from $^{15}NH_4$ tracer addition in tidal freshwater marshes demonstrated the theory that nitrogen cycling in these systems is relatively closed (Neubauer et al., 2005), evidenced by high rates of internal recycling (via nitrification; Bowden et al., 1991; Veuger et al., 2007) with small exchanges of nitrogen between the marsh and tidal waters (Gribsholt et al., 2005 and 2006). The benthic microbial community has been recognized as the most important mechanism for nitrogen retention long-term in tidal freshwater marshes (Gribsholt et al., 2007; Veuger et al., 2007; Gristhbolt et al., 2009). Other experimental additions of $^{15}NH_4$ have helped to confirm previous observations in relatively unexplored topics such as subsidies from aquatic to terrestrial habitats; for example, Sanzone et al. (2003) demonstrated that invertebrate insectivores (such as spiders and odonates) facilitate energy transfer from aquatic to terrestrial habitats by consuming emerging aquatic insects along the stream edge.

K/Na$^{15}NO_3$ additions were conducted in the LINX II experiment which included 72 streams. This large-scale project provided new insights on the removal efficiency in streams with high nitrate concentrations (Böhlke et al., 2004; Mulholland et al., 2008, Hall et al., 2009b, Mulholland et al., 2009). LINX II also demonstrated that the hyporheic zones and floodplains act ass

nitrogen sinks decreasing its nitrogen removal capability with the anthropogenic alteration of streams (Hall et al., 2009b; Hubbard et al., 2010). Although denitrification is still one of the most challenging N-cycling processes to quantify, ^{15}N tracer experiments have demonstrated that a combination of hydrological (flow velocity x depth), chemical (NO_3 and NH_4 concentrations), and biological (ecosystem respiration rate) factors are the most important controls of stream denitrification with *in situ* denitrification from stream water accounting for a small fraction of the nitrate removal (≈16%; Böhlke et al., 2004; Mulholland et al., 2009). Transient storage zones (including hyporheic zones) are hot spots for stream denitrification (Zarnetske et al., 2011a, 2011b). The decline on the efficiency of denitrification as NO_3 concentration increases has also been observed in marsh ecosystems (Drake et al., 2009). ^{15}N-NO_3 additions in headwater streams demonstrated that production of autochthonous DOC represents a substantial transformation of stream N (similar magnitude that nitrification and denitrification), depending on ecosystem heterotrophy (Jones et al., 2003).The $^{15}NO_3^-$ tracer addition technique has also improved our understanding of nitrogen cycling in complex systems such as estuaries. Studies have revealed the key importance of benthos in the N-cycling of estuaries which moreover support phytoplankton demands (Holmes et al., 2000, Tobias et al., 2003), although studies examining mechanisms of N transformation, storage and export are scarce yet. The level of efficiency in N cycling, sediment sequestration and efficiency of plant uptake in estuaries decrease with high NO_3 concentrations (Drake et al., 2009). Finally, $^{15}NO_3$ additions have also shed light on assimilation and transfer by the microbial loop in lakes (Epstein et al., 2012)

Organic nitrogen compounds have been used to assess the effects of cyanobacterial blooms on the food web of eutrophic lakes. Recently, experiments undertaken in Lake Taihu (China) using detritus from *Microcystis*

cultures enriched with ^{15}N demonstrated the key role of cyanobacterial detritus from blooms as food source for both, planktonic and benthic food webs, including quick assimilation by submerged and emergent macrophytes (Li et al., 2010; Zhan et al., 2010; Yu et al., 2013).

2.5 METHODOLOGICAL CONSTRAINTS OF ^{13}C AND ^{15}N TRACER ADDITIONS AT WHOLE-ECOSYSTEM SCALE

The success of a SI tracer addition experiments depends on the amount of ^{13}C or ^{15}N to be introduced into the system ($\Delta\delta_n$ = enriched δ – basal δ) which must have a large enough *target enrichment* to be unambiguous and measurable (immediate, large and transient increase of the isotopic signal), but simultaneously avoiding alteration of the natural concentration of the added substance (Mulholland et al., 2000a; Tank et al., 2000b; Cole et al., 2002). The *target enrichment* may involve some restrictions on the compound to be used, as well as on the amount of SI needed to obtain a distinctive signal for some end products (e.g. the use of NH_4 to trace denitrification; Hamilton et al., 2001). Obviously, detectable amounts of the SI added depend on pool sizes, pool concentrations and the number of intermediate biogeochemical transformations before the target item (Boschker and Middleburg, 2002). ^{13}C has lower fractionation than ^{15}N, and therefore, lower enrichments are needed (but not the amount of the compound to be used; Table 2.1). In fact, if ^{13}C added as inorganic carbon, there is a rapid loss into the atmosphere, and if the zooplankton community or planktivorous fish are the targets, large amounts of SI need to be added (Cole et al., 2002). In salt marshes $DI^{13}C$ additions can only be done on small scales or in some constrained environments because whole-ecosystem scale experiments face to dilution from seawater bicarbonates

(Amber, 2009, Amber et al., 2010). In complex ecosystems such as estuarine tidal flats, the amount of ^{13}C to be added is usually estimated based on previous pilot experiments in order to discover at which *target enrichment* organisms are affected (e.g. Middleburg et al., 2000). Since organic ^{13}C compounds (enriched or with distinctive signature) are used to study the importance of benthic pathways on ecosystem functioning, the amount of SI must guarantee that bacteria are labelled (Van Oevelen et al., 2006a, 2006b; Veuger et al., 2006; Bartels et al., 2012). The selection of any natural organic substances such as POC in benthic food-web experiments depends on the contrast achieved between signatures under consideration (e.g. corn starch: -10.4‰ *vs*. particulate organic matter: -28.8‰; Bartels et al., 2012) and the insolubility of the compound, which must settle quickly in order to be available to the benthic community alone. To date, only the effects of simple substrates have been simulated using ^{13}C additions, whereas the reproduction of complex substrates likes detritus using SI will have to wait.

The huge enrichment targeted in ^{15}N additions compared to ^{13}C is usually justified by the high risk of loss and dilution. In $Na/K^{15}NO_3^-$ tracer studies of denitrification, dissolved N_2 in surface waters continually exchanges with atmospheric N_2, and therefore background level and dynamic turnover are high. Consequently, elevated ^{15}N enrichments are difficult to obtain and *in situ* addition experiments commonly involve only discreet increases in $\delta^{15}N_2$. Fortunately, methods have been optimized to quantify the ^{15}N tracer at enrichment levels that may be only a few ‰ (Hamilton and Ostrom, 2007). The use of one enriched compound versus another depends on the main pools to be labeled, the total amount needed to sufficiently enrich the isotope signal, as well as economic and practical restrictions. For example, in denitrification

essays $^{15}NO_3^-$ addition can be counterproductive when nitrate concentration in the water body is very high.

The addition method varies greatly according to the needs of each tracer study and ecosystem considered. Time of addition depends on the isotopic equilibrium to be reached for selected target components, considering that most of the isotope added is lost. While instantaneous addition is less common, gradual or short pulse labelling (over a few hours) is adequate for tracing short–time scale processes such as nitrification (Tobias et al., 2001; Mulholland et al. 2009) and for water bodies with long retention times and low dispersion (e.g. Cole et al., 2006; Pace et al., 2007). Continuous addition (lasting several weeks) is used to trace the flow to the consumers in a food web in order to achieve greater and more sustained labelling (Pace et al., 2004; Hamilton et al., 2004).

To successfully trace the label and construct a reliable budget for the whole ecosystem, a prerequisite is an even distribution of SI tracer throughout the study. Many studies do not show any data for the conservative tracer addition although they cite its use. NaCl is the most available compound and is easily measured with a simple conductivity meter. Its use in aquatic systems is limited by discharge: you cannot physically add enough dissolved salt when te stream gets large. Other conservative compounds such as NaBr or fluorescent dye traces (e.g. rhodamine WT) which are easier to handle in the field, can be measured by ionic chromatography or fluorimeters whereas the use of selective electrodes is notoriously problematic. However although widely used have their own issues: Br can be toxic at elevated concentration and can alter the rate of biogeochemical cycling by inhibiting microbial activity whereas RWT may not remain conservative due to binding with organic sediments or photo-degradation (Lin et al., 2003).

A weakness of the tracer SI approach is that the isotopic values of consumers may lag behind those of their food, with the lag period being a function of the turnover rates within the consumer (Weidel et al., 2011). Although in-depth discussion on this topic falls outside the scope of this study (see Martínez del Río et al., 2009 for further information), some considerations are key for a correct assessment of food web dynamics in whole-ecosystem scale experiments using SI additions. The isotopic turnover rates and the factors that influence them must be considered in order to (*i*) determine the time window through which tissues reflect dietary isotope change, and (*ii*) assume that variability on resource use can be related in tissues with different turnover rates (Martínez del Rio et al., 2009). Results of experiments on whole-ecosystem ^{15}N tracer addition for food web dynamics would seem to indicate that the higher the N turnover rates (in the order of several days) in consumers, the more accurate the estimates of food resources (Mulholland et al., 2000b) due to the temporal scope of this experimental approach. Another constraint is that the consumer must be close to isotopic steady state (equilibrium) with respect to its diet before the isotopic enrichment of a consumer can be compared with that of its potential food at a particular point in time. Whether isotopic equilibrium has been reached in the system after SI addition is difficult to determine because each nitrogen and carbon pool needs a dissimilar length of time to reach this new labelled steady-state, especially when the degree of isotopic enrichment varies over time due to continuous ^{13}C or ^{15}N additions (Tobias et al., 2003; Hamilton et al., 2004). Variable tracer enrichment often occurs in tidal systems and, to a lesser extent, in streams because changing discharge and ambient nutrient concentrations can cause the isotopic enrichment to vary, even if the rate of isotope addition is kept constant (Hamilton et al., 2004). Variable enrichment may also result from periodical SI additions as sometimes occurs in mesocosm experiments. It is particularly

difficult to determine whether isotopic equilibrium has been reached in isotope addition experiments involving macrofauna, especially when the degree of isotopic enrichment varies over time (Lee et al., 2011).

2.6 EASE OF INTERPRETING THE RESULTS OF SI TRACER ADDITIONS AT WHOLE-ECOSYSTEM SCALE

A comprehensive review of analytical tools for analyzing stable isotope results falls outside the scope of this work (see for example, for food-web structure, Layman et al., 2012). Evidently, there is no single standard method to assess carbon and nitrogen fluxes after ^{13}C and ^{15}N whole-scale additions, and many approaches have been applied according to the manipulated and target pools, as well as the physical and biological features of each ecosystem (Carpenter et al., 2005). To date, compartmental models and linear mixing are the most commonly used analytical approaches at whole-ecosystem scale ^{13}C and ^{15}N additions (≈ 90%). Due to the dynamic nature of this approach, steady-state mixing models, like those used in studies of natural isotope abundance, are not appropriate (Carpenter et al., 2005); however, some authors studying N-cycling have used this approximation to obtain variable daily inputs (e.g. Hall et al., 1998; Böhlke et al., 2004). Tracer addition assays whose experimental design includes circular statistics (Zar, 1996) are becoming widely used to quantify the overall effects of SI addition on stable isotopes (Bartels et al., 2012) because they provide a quantitative understanding of complex isotopic changes at the community and food web levels in time and/or space (Schmidt et al., 2007).

Compartmental modelling is the most common analytical solution used in SI tracer addition at whole-ecosystem scale, mainly studying N dynamics (Hall

et al., 1998; Tank et al., 2000b; Carpenter et al., 2005; Pace et al., 2007; Solomon et al., 2008; Weidel et al., 2008). Compartmental models employ the mass-balance approach for a high number of pools simultaneously using many field measurements of the ecosystem process rates and many assumptions on ecosystem structure and feedbacks (Cole et al., 2002). Compartmental models have to be developed for each particular process to meet both the characteristics of each ecosystem type (biotic *versus* lentic) and the aims of each essay but provide a detailed dynamic analysis (more fluxes among ecosystem compartments than other models), grounded in current understanding of the major processes governing nutrient flows in ecosystems (Cole et al., 2002). This approach enables dynamic assessment of trophodynamics and the description of interaction and energy flow between organisms and compartments (Pimm, 2002). One of the first attempts at compartmental modelling in SI tracer addition was developed by Hall et al. (1998) on studying N-cycling in streams, but did not consider any transformation rates. The most comprehensive compartmental model for lakes was the dual-isotope flow modelling (DIF, Cole et al., 2002), which employed mass-balance of total carbon and ^{13}C for 12 carbon pools with many pool sizes and flows directly measured to calibrate the model. The study of stream denitrification requires more complex compartmental models based on exhaustive reaction rates, which include solute travel times, variable air-water gas exchanges affected by temperature and other gas fluxes, and reach-averaged rates of groundwater discharge, nitrification, and NO_3 assimilation (Böhlke et al., 2004; Mulholland et al., 2004, 2008). The main weakness of these models is the use of steady-state reaction parameters to simulate stream data, which is often justified because the storage zones are the main reaction sites in streams. Although results are usually very realistic, since they depend on a large number and diversity of measurements, potentially complex errors

can result (Carpenter et al., 2005). Compartmental software such as Simulation Analysis and Modelling (WinSAAM, http:// www.winsaam.org; Hamilton et al., 2004) has been developed to simulate and fit data, and to model the transfer rate of tracers in a system, without requiring the system to have reached a steady state. Although its use in SI tracer additions at ecosystem-scale is very recent (C flow in food webs; Lee et al., 2011), results are promising given the implicit simplicity of the model.

Besides the linear mixing models like those used in food-web studies (e.g. Middleburg et al., 2000; Kritzberg et al., 2006; Vanden Meerche et al., 2011), dynamic mixing models have been used in several SI tracer addition experiments. The univariate time-series model (UNI) developed by Pace et al. (2004) and Carpenter et al. (2005) is a simple parsimonious analytical tool with few assumptions predicting the response of one pool at a time from one isotope manipulation, using standard statistical methods (i.e., only attempt to model the response of a single variable as a function of two sources). The main weakness of UNI is that it overlooks information on the dynamics of closely related time series and does not attempt to represent the specific ecological processes governing carbon and nitrogen flows (Carpenter et al., 2005). Dynamics of $\delta^{13}C$ in slowly changing pools (benthos or fish) are poorly predicted using UNI from the relatively rapid changes of $\delta^{13}C$ in DIC and the many transformations occurring as carbon moves through the food web to these consumers (Carpenter et al., 2005). Multivariate autoregression (MAR) models (Ives et al., 2003) provide an intermediate solution, which also employs isotope time-series from the source to consumer compartments but also incorporate some additional information by including the dynamics of closely related variables (Carpenter et al., 2005). Both UNI and MAR models present a good agreement and differences with DIF model estimates, possibly representing more realistic

results, have been related with model uncertainty (Carpenter et al., 2005). Linear mixing models have been extended to account for uncertainty in source partitioning (Isoerror: Phillips and Gregg, 2001) and concentration dependence (Isoconc: Phillips and Koch, 2002).

Software such as IsoSource was designed because most food webs are too complex to use simple linear mixing models (Layman et al., 2012). IsoSource is a mixing model designed for situations in which *n* isotopes are being used and more than *n + 1* sources are likely to contribute to a mixture, using all possible resource combinations to determine a range of possible source contributions (i.e. minimum and maximum possible; Phillips and Gregg, 2003). The isotope values of each mixture are constrained by isotopic mass balances (Phillips, 2001) but the range values for each source contribution emphasize the absence of a single solution, requiring the use of additional qualititative information to further sort the results (Benstead et al., 2006). IsoSource has been most useful, showing that sources are not as important for a food web as computing source contributions (Taipale et al., 2008; Galvan et al., 2012).

Finally, Bayesian mixing models, used mainly in food-web studies, are one of the most recently developed approaches (Solomon et al., 2011; Holtgrieve et al., 2010). Although still under improvement, they are able to incorporate $\delta^{13}C$ and $\delta^{15}N$ from multiple dietary sources and generate potential dietary solutions as true probability distributions; examples of Bayesian mixing models are MixSir (Moore and Semmens, 2008) and SIAR (Stable Isotope Analysis in R; Parnell et al., 2010; Solomon et al., 2011). To our knowledge no study of SI addition at whole-ecosystem scale has used this type of approach yet. Compartmental models are difficult to analyse for uncertainties and less accurate than dynamic mixing and Bayesian models.

Stable isotope data can also be incorporated into ecological network analysis and simulation software to assess and identify system interactions and holistic properties (Fath et al., 2007). EcoNet (http://eco.engr.uga.edu/index.html; Kazanci, 2007) uses network environmental analysis, deterministic and stochastic algorithms, to quantify the actual relationship between compartments, environmental inputs and outputs (Tollner and Kazanci, 2007; Ings et al., 2009). The SI tracer experiment of Lee et al. (2011) combined the software WinSAAM to quantify C flow by comparing the temporal patterns of producer and consumer ^{13}C enrichment and EcoNet assess food-web dynamics by providing system indices that quantify the flows and food-web interactions. This interesting approach allows concurrent quantitative assessment of food web structure and ecosystem functioning independent of isotopic equilibrium.

2.7 MAIN RESTRICTIONS IN THE USE OF ^{13}C AND ^{15}N AS TRACERS TO STUDY AQUATIC PROCESSES AT WHOLE-ECOSYSTEM SCALE

There are still many practical, methodological, and interpretative limitations that restrict aquatic scientists' widespread use of SI tracer additions as a complementary tool. The first basic constraint of using ^{13}C and ^{15}N enrichments at ecosystem scale is related with the *in situ* nature of field experiments. This means results are more variable and unpredictable than laboratory experiments: the predicted assumptions may not always be met or the results are often difficult to interpret. However, this *in situ* approach reflects a more realistic view of the system. The first concerns the fact that processes other than those targeted by the experiment can alter the distribution of SI in the ecosystem, potentially adding uncertainty to quantitative

interpretation of results; $\delta^{13}C$ in a given carbon compartment is expected to change due to ^{13}C addition and to other uncontrollable factors, such as fractionation (Pace et al., 2007). In fact, numerous practical and methodological questions related with fractionation processes in nature remain unsolved (see Martínez del Río et al., 2009; Kelly and Martínez del Rio, 2010). Have enough enrichment in the pool of the interest, making any potential fractionation trivial is the main point of adding the tracer. An inherent limitation of SI tracer addition in aquatic ecosystems is related to isotopic equilibrium: whether isotopic equilibrium has been reached in each pool during SI addition is difficult to determine because each nitrogen and carbon pool needs a dissimilar length of time to reach this new labelled steady-state, especially when the degree of isotopic enrichment varies over time due to continuous ^{13}C or ^{15}N additions (Fig. 2.3; Tobias et al., 2003; Hamilton et al., 2004). Problems occur especially with larger bodied organisms with nutrient pools that turnover slowly (Tank et al., 2000b). This constraint can now be partially avoided using analytical solutions that do not require isotopic equilibrium (Lee et al., 2011).

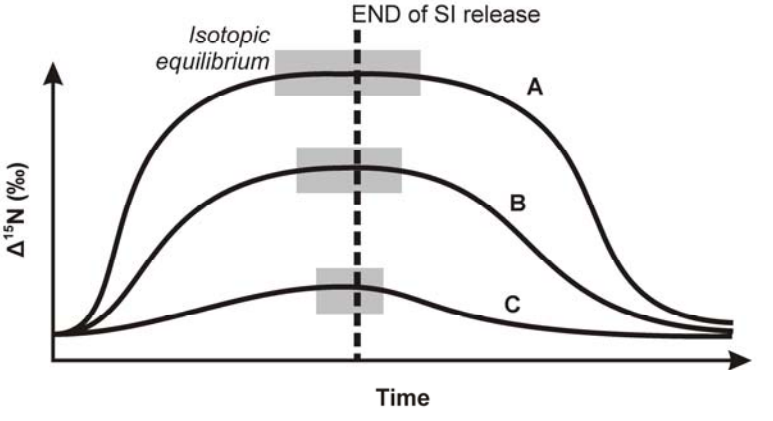

Fig. 2.3: Dissimilar isotopic equilibrium in three different pools which were traced during one single SI addition experiment. The length of time to reach this new labelled steady-state and the time during which each pool is enriched varies.

The uncertainty of estimates represents one of the main challenges facing the models used to interpret SI data from tracer experiments. Although it is a very important aspect, it should not be the only consideration when working with variables and stochastic processes that we intend to study from a mechanistic point of view. The fact that compartmental models provide the most robust results is evidenced by their widespread use in SI tracer addition experiments, but requires uncertainty estimates to improve their reliability. However, so far, neither dynamic mixing nor Bayesian models have achieved the mechanistic needs of many complex biogeochemical studies. A combination of both approaches may provide solution to this issue.

Limitations also concern the absence of standardized protocols for sample collection and preparation for SI analyses whereas measurements (IRMS, MIMS) are quite similar. SI tracer additions at whole-ecosystem scale are very complex experiments involving numerous sampling in several compartments simultaneously, which can include dissolved and particulate substances as well as biotic samples. This disparity often leads to the use of alternative methods to better fit the circumstances of each experiment but make it hard to compare results. Only a few stable isotope laboratories have standardized protocols for each sample type preparation (e.g., Colorado Plateau Stable Isotope Laboratory at the Northern Arizona University, http://www.mpcer.nau.edu/isotopelab/and Stable Isotope Facility at University of California, www.stableisotopefacility.ucdavis.edu/). Protocols for sample collection and pre-treatment, such those developed in LINX (http://www.faculty.biol.vt.edu/webster/linx/) could help improve comparison between isotope results. More SI addition experiments sharing results and methodologies would encourage the development of protocols applicable to each type of aquatic system.

Finally, we are all aware of the laborious efforts and expense required for SI addition experiments, which might discourage the development of this field. Intensive field campaigns, high sampling frequency, large number of samples to be prepared and analyzed and high analytical prices due to the elevated costs of analyses, laboratory operation and supplies. These economic restrictions often limit the feasibility of tracing isotope transfer to the upper trophic levels or of investigating temporal trends in aquatic processes. Although ^{15}N addition provides the most comprehensive set of stream denitrification measurements to date, they only represent a snapshot in time in each stream; thus, data cannot be used to address the issues of seasonal or longer term variations in denitrification rates (Mulholland et al., 2009; Peterson, 1999; Gribsholt et al., 2007). The high costs involved in these types of SI experiments mean that the assays can only be programmed for very short time periods, leading to results which are not very representative of the mean annual rates at which aquatic ecosystem processes operate (Mulholland et al., 2009).

2.8 FUTURE CHALLENGES OF 13C AND 15N TRACER ADDITION EXPERIMENTS TO SOLVE CURRENT AQUATIC ECOLOGY AND BIOGEOCHEMISTRY PARADIGMS

Since the first seminal *in situ* SI tracer addition experiment carried out by Kling (1994), ecological and biogeochemical concerns and paradigms have changed and studies have been modified to solve current environmental and ecological challenges. Today, one of the biggest challenges facing aquatic ecology and biogeochemistry concerns the role of the microbial communities sustaining ecosystem functioning (Hall and Meyer, 1998). The metabolic rates and dynamics of nutrient transformations in the nature are not well known yet

(Dumont and Murrell, 2005; Jetten et al., 2009). Although ^{13}C and ^{15}N enrichment experiments are potentially able to solve most uncertainties in aquatic microbial ecology, a greater effort is needed to develop new methods and techniques to improve current results. In fact, SI tracer addition cannot be used everywhere. For example, we currently have no good method to use SI in larger streams and rivers which ensure that SI addition is enough to label benthos sufficiently and to determine the long-term fate of the assimilated ^{15}N (O'Brien et al., 2012). The use of stable isotope-labelled substrates in combination with biomarkers has allowed quantification of degradation rates and identification of organisms involved (Bull et al., 2000). Specifically, some processes, such as denitrification, require further investigation, as do other recently described processes like anammox, dissimilatory nitrate reduction to ammonia (DNRA) and denitrification by sulfur-oxidizing bacteria (Mulholland et al., 2008 and 2009; Burgin et al., 2012). Compound-specific isotope analysis, as well as combining the SI tracer addition approach with quantitative PCR of denitrification genes (e.g. Findlay et al., 2011; Vilar-Sanz et al., 2013) would provide a wide range of possibilities to study these complex microbial transformations in the aquatic environment (Boschker and Middleburg, 2002; Johnson et al., 2012). Future assessment of other ecological assumptions that are still open to debate may be supported by the results provide by experiments using ^{13}C and ^{15}N as tracers, for example: the role of allochthonous C inputs in lakes according to their environmental properties (Pace et al., 2007); the effects of increased N loads in lakes on nitrogen cycling and food web structure (Pace et al., 2007); the importance of wetlands and hyporheic zones in the nitrogen transformation at landscape scales (Duff and Triska, 2000; Böhlke et al., 2009); the metabolic support of food webs; the N uptake rates and control variables in benthic regions (Wetzel, 2001); or the seasonal variability of food web structure because of changes in biotic (basal resources, reproductive or stages

of development) and abiotic (temperature, runoff, rainfall or evaporation) constraints (Layman et al., 2012).

In natural abundance studies, dual-isotope approaches (simultaneous study of ^{13}C and ^{15}N) is a common practice to assess trophic pathways in aquatic systems (Yoshii et al., 1999; Post et al., 2002; García et al., 2006; Bratkič et al., 2012), while multi-isotope approaches (combining more than two SI) may represent a potential advance in aquatic research (e.g. the combined use of ^{15}N, $N^{18}O_3$ and $N^{17}O_3$ is a promising tool to determine nitrate sources and reactions; Kendall et al., 2010). In SI addition experiments, simultaneous enrichments are still seldom used. Including a second tracer drastically increases both the logistics and expenses, which are a major part of the reason few studies have attempted it. Although, only a few experiments at ecosystem-scale have taken advantage of these simultaneous enrichments (^{13}C + ^{15}N), their results were highly promising in terms of elucidating organic matter sources, food resources and trophic patterns (Mulholland et al., 2000b; Carman and Fry, 2002; Parkyn et al., 2005; Taipale et al., 2008; Amber et al,. 2010; Vanden Meerche et al., 2011; Galván et al., 2012); therefore, they should promote more dual isotope addition studies, at least in small water bodies.

Smaller sample size and faster sample processing in addition to lower sample cost have also led to the use of isotopes in studies of ecosystem processes (Hobbie et al., 1999). New technical methods as stable isotope probing (SIP), secondary ion mass spectrometry (SIMS), 13C-nuclear magnetic resonance or tunable diode laser absortion spectroscopy (TDLAS) are providing new perspectives to the use of SI in ecosystem studies (see Sulzman, 2007). Several attemps have been done to directly coupled HPLC with IRMS, but commercial machines are not available yet and sensitivity is still rather

low; HPLC-IRMS would greatly broaden the types of biomarkers that can be analyzed (Boschker and Middleburg, 2002).

Finally, progress must be made to solve the isotopic equilibrium. Recent advances in modelling approaches provide a robust estimation of carbon and nitrogen dynamics that can deal with deviations from the isotopic equilibrium (e.g. end members, sources, trophic enrichment and isotopic routing factors) in order to minimize mistaken inferences in data analysis and results (Martínez del Río et al., 2009; Parnell et al., 2010). Aquatic ecosystems are stochastic systems, and biogeochemical processes need to be assessed through mechanistic models; therefore, compartmental models built from a Bayesian perspective could increase the accuracy of SI addition data, handling uncertainty as a variable that improves the robustness of results. Approaches like compartmental models combined with ecological network analysis, which allow quantitative assessment of the food-web structure and ecosystem functioning, are examples of the way forward for this type of modelling.

2.9 CONCLUSIONS

In last two decades, SI tracer addition experiments at ecosystem scale have been used widely in aquatic environments. This period has witnessed an improvement in both the procedures and compounds used to modify the desired pools and achieve specific goals; there has also been progress in the available analytic tools. Today SI tracer addition at whole-ecosystem scale can be considered a powerful complementary tool to study ecological and biogeochemical processes at whole aquatic ecosystem scale. This approach has improved our knowledge of food-web ecology and carbon and nitrogen biogeochemistry, shedding light on the structure and functioning of aquatic

ecosystems. However, some methodological and theoretical constraints hinder the wider use of this approach by aquatic scientists. Some of these limitations can be easily solved by trial and error, such as the most appropriate compound to be used according to the experiment goal, the target enrichment or the timing of injection. Although modelling approaches required to interpret isotopic results have improved substantially during recent decades, in most cases data from SI tracer addition experiments at ecosystem-scale in aquatic environments need to be analysed using a compartmental modelling approach. This is because specific pools and rates need to be incorporated in order to reflect the main features of each ecosystem mechanistically. Therefore, data interpretation from SI tracer experiments is still subject to great uncertainty. While dynamic mixing models are not able to properly reflect the stochasticity of most natural processes traced using SI, other recently developed approaches based on Bayesian statistics can improve the accuracy of our estimates. The temporal representativeness of results, considering the short duration of SI tracer addition experiments, remains a serious problem in this context.

In ecology and biogeochemistry, the *in situ* nature of this field approach represents a strong advantage instead of an apparent limitation. Clearly ^{13}C and ^{15}N enrichment experiments at ecosystem scales require laborious effort and are expensive but not much more than other traditional approaches. It is reasonable to expect that laboratory equipment and methods of SI analysis will improve in the near future, thereby lowering costs and saving time.

Numerous concerns and paradigms on aquatic ecology and biogeochemistry can be assessed successfully using ^{13}C and ^{15}N enrichment experiments at ecosystem-scales. Metabolic rates and dynamics of microbial processes involved in aquatic N and C cycles are not sufficiently well known and represent one the main challenges facing this approach in the coming years.

More multi-isotope tracer experiments at whole-ecosystem scale should be performed as this simultaneous use has numerous advantages which could bring about a radical change in the potential of this approach, promising remarkable progress in aquatic sciences.

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CHAPTER 3

Testing nitrogen dynamics in a stream influenced by agriculture: ^{15}N tracer addition approach

"*Streams and rivers are not merely systems for moving surface water to the world's oceans and seas, they form vital links in the global biogeochemistry cycle*"

Walter K. Dodds

3.1 SUMMARY

In streams, ^{15}N addition tracer tests are a tool commonly used to understand not only the concept of nutrient spiraling, but also other relevant aspect of N cycling in these aquatic systems. Human activities have altered the nitrogen (N) cycle in streams so deeply through increased wastewater discharge, the over-use of fertilizers and overgrazing practices, that knowledge of N processes has become increasingly desired, particularly in agricultural streams. In this study, we assessed main ammonium transformations in a small 3rd order low-land agricultural stream (Arroyo Grande del Molinillo) by using a short time whole-scale addition of NH_4 and $(^{15}NH_4)_2SO_4$. To this purpose, we calculated whole-system and compartmental ammonium uptake rates, nutrient spiraling parameters and reach-averaged rates of N flux of ^{15}N-NO_3, ^{15}N-NH_4, ^{15}N-N_2, ^{15}N-N_2O. Our results showed long ammonium spiraling lengths, relatively low uptake velocities and a high residence time, suggesting poor NH_4 retention efficiency in the study reach. We observed low rates of nitrification, suggesting that the NH_4 in the studied stream may have exceeded a threshold, causing a decrease in NH_4 biological demand via nitrification. Furthermore, our results also highlighted the importance of the hyporheic zone as a key site for nitrogen metabolism.

Keywords: isotope enrichment, agricultural stream, stream-uptake rate, nutrient spiraling, nitrification saturation

3.2 INTRODUCTION

Along the land-to-ocean aquatic continuum, streams and rivers have been viewed not only as conduits for nitrogen (N), but also as key sites for N storage, transformation and removal (Martí and Sabater, 1996; Alexander et al., 2000; Peterson et al., 2001). Regional budgets show that only about 10–25% of N added to land is exported to the ocean, indicating that inland freshwaters are important sinks, affecting the amount of N that is transported (Billen et al., 1991; Howarth et al., 1996; Boyer et al., 2002; Schaefer and Alber, 2007). Human activities have altered these N dynamics through increased wastewater discharges, the over-use of fertilizers and over-grazing practices, being lowland streams located in agricultural catchments especially affected (Vitousek et al., 1997). Agriculture accounts for ~ 86 % of anthropogenic N inputs to the global N cycle (Vitousek et al., 1997). Galloway et al. (2004) estimated that ~ 50% of the N entering streams and rivers may be removed before it reaches coastal waters; however, many streams have been so heavily impacted by human activities that they cannot appreciably reduce in-stream N (Bernot and Dodds, 2005). As a consequence, over the past decade, there has been substantial interest in examining N compounds and fluxes in streams and rivers over broad geographic regions and under different environmental settings in order to predict transformations and fates (e.g., how rapidly inorganic forms of N are immobilized and which components of the ecosystems are most important in the initial uptake and log-term retention; Mulholland et al., 2000; Sanzone, 2001).

Chemical form in which N is present in an aquatic ecosystem has a wide range of ecological consequences, defining the N cycle in the ecosystem. The

dissolved inorganic pool (DIN), ammonium (NH_4) and nitrate (NO_3), that enter into the stream is directly incorporated by aquatic primary producers (Ashkena et al., 2004) or temporary stored in the ecosystem through biotic and abiotic sequestration from days to years before being again released (Bernot and Dodds, 2005; Findlay et al., 2011). DIN can be microbially transformed, through nitrification and dissimilatory nitrate reduction to ammonium (DNRA), and still remaining in a biologically available N forms (Ensign and Doyle, 2006; Smith et al., 2007; Bartrons et al., 2010). DIN can be removed from the ecosystem via anammox and denitrification (Penton et al., 2006; Findlay et al., 2011; Fig. 3.1). These processes are ultimate responsible of defining the rates of N retention and removing, which is an insightful measure of lotic ecosystem functioning (Wetzel, 2001; Dodds, 2002).

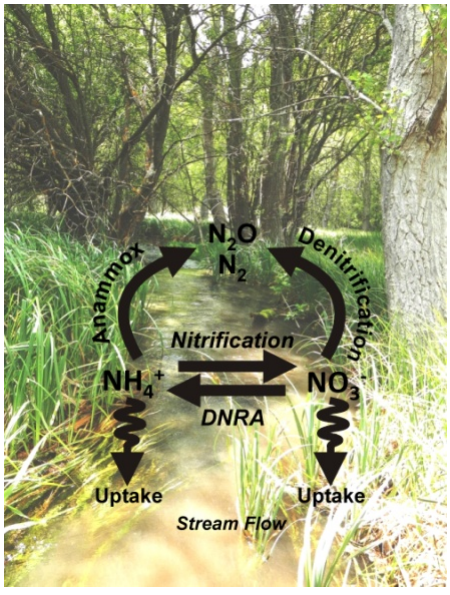

Fig. 3.1: Dissolved inorganic nitrogen (DIN) dynamics in a fluvial ecosystem.

In streams all these processing rates are closely related to the concept of "Nutrient Spiraling" (Webster, 1975; Newbold et al., 1981, 1982), which involves nutrient cycling and downstream nutrient transport (Webster and Patten, 1979; Elwood et al., 1983).

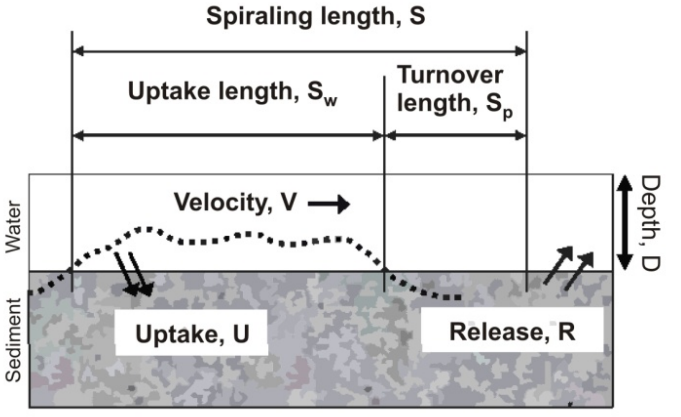

Fig. 3.2: Two compartment nutrient spiraling model (in Newbolt et al., 1982).

This approach means that each nutrient molecule, such nitrogen, is in the water column for an average amount of time while it moves downstream (Fig. 3.2). It then is taken up by an ecological component of the stream until it gets back to the dissolve inorganic phase again (Newbold et al., 1981; Stream Solute Workshop, 1990). Thus, the nitrogen cycle that is conceptualized as a wheel in lakes becomes a spiral in streams (Dodds, 2002). This concept has some practical weaknesses, such the strong influence of temperature and hydrological factors in uptake length values, allowing comparison only among streams with similar environmental attributes (Aumen et al., 1990; D'Angelo and Webster, 1991; Martí and Sabater, 1996). Therefore, the nutrient spiraling approach has being viewed for a long time as a qualitative measure of nutrient retention efficiency of an ecosystem (e.g.,

streams with short spiraling lengths are considered more efficient in retaining nutrients than streams with longer ones, Newbold et al. , 1981, 1982; Elwood et al., 1983), whereas quantitative information was constrained by the availability of accurate methods (Peterson et al., 1997; Bernot et al., 2003; Kulkarni et al., 2008).

^{15}N tracer approaches provide the study of ecosystem nutrient dynamics under ambient conditions, without the artefacts resulting from simulation of processes rates (Mulholland et al., 2008; O'Brien et al., 2007; Peterson et al., 2001). Indeed, the use of ^{15}N tracer additions have enabled stream ecologists to study internal N cycling, nutrient uptake rates and lengths in a variety of streams, including tundra (Peterson et al., 1997), forest (Hall et al., 1998; Mulholland et al., 2000; Tank et al., 2000; Ashkenas et al., 2004) and prairie streams (Dodds et al., 2000). The dominant source of information regarding whole-stream N-cycling came from the Lotic Intersite Nitrogen eXperiments (LINX I and II), a multi-site study of nitrogen uptake and transformations by means of ^{15}N tracer additions carried out in different aquatic settings and geographic regions throughout the United States (Mulholland et al., 2002; Hall et al., 2009). Even though the understanding of N dynamics in streams has been advanced considerably by these whole-stream experiments (e.g. Mullholland et al., 2008), most information proceed from streams receiving low nutrient discharges, and there is still a need for further understanding of the processes controlling nitrogen dynamics, particularly in streams influenced by high NH_4 and NO_3 concentrations (Riis et al., 2012). In streams located in agriculture catchments, studies have been mainly focused on the role of the increased nitrate concentration on denitrification, but processes related with

other compounds as ammonia have been neglected and we unknown how ammonia is processed under these environmental conditions.

In this study we integrated conceptually and experimentally several aspects of the N cycle in a stream, combining hydrodynamics and metabolism measures with nutrient and isotope tracer additions. We used NH_4 and $^{15}NH_4$ as tracers to follow the uptake, transformations, retention and export of N-NH_4, describing N cycling in a small stream influenced by agriculture land use. We hypothesized that in stream influenced by agriculture, the high nitrate loads saturated the metabolic pathway of nitrification, having effects on the ecosystem functioning through nutrient availability.

3.3 MATERIAL AND METHODS

Study site

The study was conduct in a third order stream, Arroyo Grande del Molinillo, in the Tajo River basin (Fig. 3.3), located under the semiarid climate (average annual rainfall 483 mm y^{-1}, mean air temperature 14.7º C for the period 1948-2014), in central Spain (Toledo province: 40°03'30"N 4°25'26"W). This stream drains a 215 km^2 arkosic sandstone watershed dominated by rain-fed agriculture lands. The experiment was carried out in a 180 m stream reach, which is located inside La Higueruela Experimental Farm (MNCN-CSIC). The study stream reach is an open canopied segment surrounded to the south by poplars (*Populus alba*) which were afforested 30 years ago in order to drain the terrain during flooding.

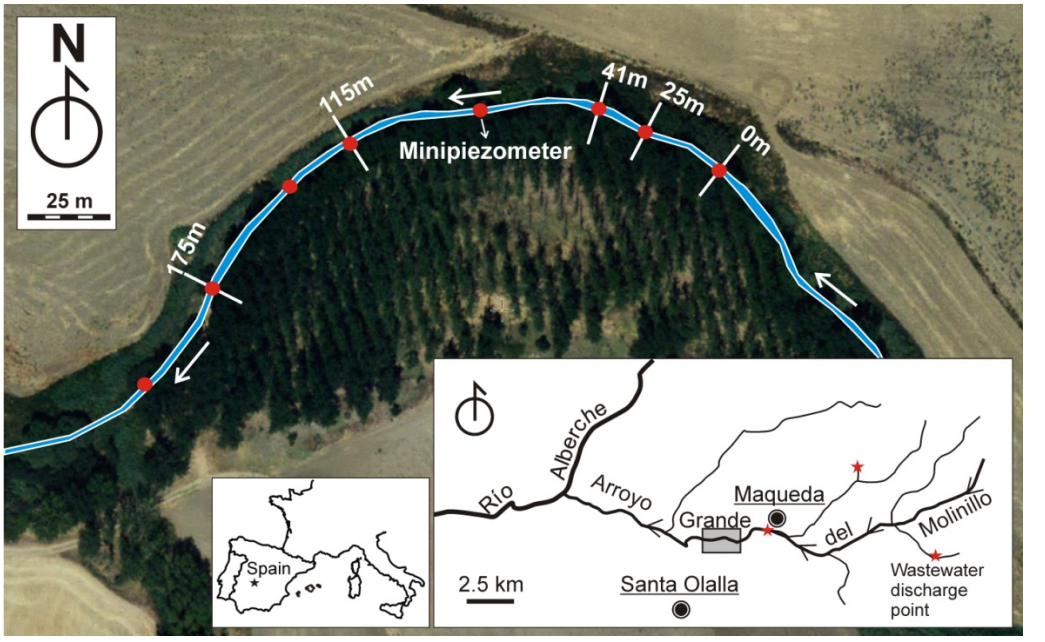

Fig. 3.3: Location map and schematic diagram of the stream section.

The alluvial plain cross section shows a strong asymmetry with the channel located to the right margin and an extensive floodplain in the left margin. River vegetation is composed mainly by *Typha latifolia*, *Typha angustifolia*, *Arundo donax, Rubus sp* and *Alnus sp.* Channel width ranges 3.5-5.5 m with depth oscillating 0.3-0.6 m. The natural stream flow regime is altered by wastewater discharges of 4 towns located upstream (50-80% of the overall stream flow), increasing the flow during the dry season and maintaining relatively high nutrient concentrations (Table 3.1). Although nitrate amount in this stream is still below the limits for being considered as a contaminated watercourse (5.6 mg/l N-NO_3; Nitrate Directive 91/676/CEE), concentration is elevated and nitrate is the main pool in the dissolved nitrogen puddle.

Table 3.1: Physical, chemical and ecological attributes of surface and sub-surface water in Arroyo Grande del Molinillo. (*) not measured.

	Sub-surface	Surface
Stream caracteristics		
Depth (m)	*	0.3-0.6
Width (m)	*	3.5-5.5
Flow velocity (m/s)	*	0.12
Discharge (l/s)	*	70-112
Mean GHV	0.05	
K_h (cm/s)	0.0032	
Water temperature (°C)	16.1	14.2
pH	7.29	8.72
Conductivity (µs/cm)	888	805
Dissolved oxygen (mg/L)	2.87	8.34
Nutrient concentrations		
TDN (mg N/l)	0.68	4.16
NH_4 (mg N/l)	0.22	0.04
NO_3 (mg N /l)	0.03	3.96
TOC (mg/L)	3.02	4.93
DOC (mg/L)	2.52	4.74
PO_4 (mg P/l)	0.76	0.83
TP (mg P/l)	0.85	0.93
Clorophyl-*a* (µg/L)	*	4.16
Metabolism (mg C/m^2/d)		
Gross primary production (GPP)	*	13.17
Ecosystem respiration R_{24}	*	105.73
Net primary production (NPP_i)	*	-92.56
GPP/R_{24}	*	0.12

Stream characterization

Hydrodynamic, metabolism and nutrient dynamics were measured approximately two weeks before the NH_4^+ and $^{15}NH_4^+$ additions. To this end, the stream reach was split in four segments: (1) 0-25 m, (2) 25-41 m, (3) 41-115 m and (4) 115-175 m (Fig 3.3).

Hydrodynamic properties- vertical potential water exchanges between the water column and the hyphoreic zone was measured using 8 minipiezometers, which consist in PVC tubes of 160 cm long and 5 cm diameter, 20 cm drilled at the base (30 holes of 3 mm diameter each) and covered by a metal mesh of 45 μm of pore size to allow hyporheic water circulates but preventing the entry of sediment. Minipiezometers were installed along the stream segments at a depth of around 60 cm in the channel bed, ensuring that the basal area was completely buried and the water flow is allowed from the hyphoreic zone (Dahm et al., 2006). Vertical hydraulic conductivity and hydraulic gradient were used to assess recharge (upwelling) and discharge (downwelling) areas as well to estimate the hyporheic water flow velocity (subsurface water; Elosegi and Butturini, 2009). Hydraulic vertical gradient (GHV, dimensionless) was estimated as:

$$GHV = h_s\text{-}h_p / L \qquad \text{(Eq.3.1)}$$

where h_s is the distance from the mouth of the piezometer to the stream water level, h_p is the distance between the mouth of the piezometer to the water surface inside the piezometer and, finally, L is the depth of the piezometer within the channel bed. L value was obtained as the difference between the total length and the non-buried length of the piezometer. h_s was measured manually with a tape from the mouth of the piezometer until the water

surface transverse to the flow and h_p was measured by using a water level meter (Nordmeyer) into the minipiezometer. The hydraulic conductivity (K_h, cm/s) was estimated using the Hvorslev' equation (1951):

$$K_h = [(r^2) * \ln (L / R)] / 2LT_{37\%} \quad \text{(Eq.3.2)}$$

where r is the radius of the minipiezometer, L is the length of the perforated portion of the minipiezometer (20 cm), R is the radius of the minipiezometer in the perforated area (in our case r = R) and $T_{37\%}$ is the characteristic time lag, that is, the time required for the level recovery until 37% of the initial water level after the minipiezometer was empty by water pumping (Elosegi and Butturini, 2009).

Microbial nitrogen content- we measured microbial N content in both, the benthic organic matter (BOM) and the hyphoreic organic matter sediments (HOM). At the time of the experiments epilithon was not present on the surface sediments, rocks or other solids. Three sediment samples in each of the four segments' stream were collected by hand with the help of a core sampler. In the field, sample was separated by 0-5 cm (BOM) and 15-30 cm (HOM) and preserved < 4° C during transport to the laboratory. Microbial N were extracted by using the chloroform-fumigation technique (Brookes et al., 1985). Briefly, 20 g (dry weight) of each sample were exposed to 50 ml of $CHCl_3$ for 24 hours to lyse microbial cells. Subsequently, the organic content was extracted with 100 ml of 2M KCl, after filtered through a Whatman GF/F and finally analyzed on a Shimadzu TOC-V coupled to a TNM-1 module. As extraction efficiency ratios of nitrogen value of 0.54 was used (Brookes et al., 1985). The biomass obtained was expressed in µg N / g dry weight (DW) of sediment. Differences on nitrogen concentration obtained between fumigated and non-fumigated samples represented microbial N.

Results from non-fumigated samples were used to estimate N standing stocks in sediment samples. Carbon content and C:N ratios of pool material from each compartments were also determined using the same technique above mentioned.

Metabolism- whole-stream rate of gross primary production (GPP) and ecosystem respiration (R) were determined using the upstream-downstream dissolved oxygen change technique (Young and Huryn, 1998) during one week period (15-22 May 2013). Two probes (AYSI 6920 and YSI proODO) previously intercalibrated were immersed at the beginning and the end of the study reach to measured dissolved oxygen at 10 min intervals. The instantaneous rate of net ecosystem production (NEP_i, g O_2/m^2 day) was performed according to:

$$NEP_i = = [(\Delta C/\Delta t) + k_{O2}(C_s\text{-}C)]\ z \qquad \text{(Eq. 3.3)}$$

where C is the dissolved oxygen concentration (g O_2/m^3), C_s is the saturation concentration of oxygen (g O_2/m^3), k_{O2} is the coefficient of reaeration (1/day) and z is the average depth of the section (m; Acuña et al., 2009). k_{O2} was calculated following Owens et al. (1964; $k_{O2} = 5{,}35\ v^{0.67}/d^{1.85}$), as suitable for shallow systems, and corrected for water temperature [$k_g(T) = k_{g(20)}1{,}024^{(T-20)}$]. The oxygen saturation concentration was also corrected as a function of temperature [APHA 1992: $Ln(Cs) = -139{,}344+(1{,}5757.10^5/T)-(6{,}6423.10^7/T^2)+(1{,}2438.10^{10}/T^3)-(8{,}6219.10^{11}/T^4)$], where Cs is oxygen saturation concentration and T is the temperature.

Metabolism was also measured in the different stream compartments: seston (water column), BOM and HOM. Same time to core collection for N microbial measurements, cores were taken and sediments transferred were

transferred to DBO bottles and incubation chambers. Seston and BOM gross production were measured by using clear and dark DBO bottles, whereas HOM respiration was measured by using opaque and hermetic metracrylate tubes (30 cm X 5.5 cm), preserving the whole intact sediment core during metabolism test. Incubation flasks were refilled of stream water, avoiding the entrance of air bubbles. All the incubations were carried out in the riverbed to maintain natural conditions, and they were performed per triplicate (3 dark and 3 clear for BOM and seston, and only 3 dark for HOM) in all the four segments along the stream reach. Incubation periods were 4 hours for BOM and 3 hours for seston and HOM to minimize the oxygen consumption/saturation risk following Wetzel and Likens (2000) and Grace and Imberger (2006). Since BOM incubations have different sediment mass amounts, bulk densities and dry weights were measured in the lab to correct final values. Changes in oxygen concentrations were measured by using a multi sonde line high pressure (HACH multi HQ40d) with a LDO sensor. Net primary production (photosynthesis) was estimated as:

$$PPN\ (mg\ O_2/m^3\ h) = (LB_{clara})-(IB_{clara})/\ t \qquad \text{(Eq. 3.4)}$$

where LB_{clear} is the final oxygen concentration in the clear bottles (mg/l), IB_{clear} is the initial dissolved oxygen concentration (mg/l) and t is the incubation time (hours). Results are expressed as mg O_2/m^3day. Respiration was computed following:

$$R = (IB_{oscura})-(DB_{oscura})/t \qquad \text{(Eq.3.5)}$$

where IB_{dark}, is the initial oxygen concentration (mg/l), DB_{dark} is the final oxygen concentration in the dark bottles (mg/l) .

Gross primary production was estimated as :

$$PPB = PPN+R \quad \text{(Eq.3.6)}$$

The heterotrophy of the system was estimated based on the P/R index ($P/R=PPB/R_{24}$); values above 1 indicated autotrophy, whereas values below 1 means the system is subsidize by external sources and, by hence the system is heterotrophic (Acuña et al., 2009). Phytoplankton chlorophyll-*a* was measured spectrophotometrically after extraction with methanol (Marker et al., 1980).

Nitrification rates- since nitrate was not traced during ^{15}N tracer additions we carried out nitrification assays in the laboratory in order to assess whether it was potentially possible and it was influenced by the effects of ammonium limitation. Nitrification experiments were performed using the nitrapyrin, which inhibits the function of the enzyme ammonium mooxygenase and hence inhibits ammonium oxidation. Several previous studies have used this method successfully to measure nitrification rates in aquatic sediments (Hall, 1984; Dodds and Jones, 1987; Strauss and Lamberti, 2000). Sediments used for nitrification tests were collected using a core sampler (0-5 cm) at the 4 segments along the stream. Water used for incubations was also collected from the stream, the same sampling day. Water was collected mid channel and was not filtered. Sediments and water were conserved < 4° C until nitrification experiments were performed. Sediment slurries were prepared for each sediment sample using fresh homogenized sediment (coarse organic debris were prior removed) and stream water until obtain an average density of varying 0.04-0.08 g/cm^3 (e.g. 250 ml of sediment in 500 ml of water). Incubations (25 ml of slurry) were prepared in duplicate and each nitrification replicate in the experiments consisted of four 250-ml flasks. One flask received only the sediment slurry with no additions (control), the second one

received NH_4^+ final concentration (0.75 mg/l NH_4Cl), to assess the effects of ammonium limiting on nitrification, the third one received a final concentration of 10 mg/L of nitrapyrin dissolved in dimethyl sulfoxide, because nitrapyrin is insoluble in water, and the last fourth one received both compounds, NH_4^+ and nitrapyrin. Flasks were covered loosely with aluminum foil to avoid light and incubated for 72 hours in the dark at constant temperature (25 °C). After the incubation period, flaks were introduced to the freezer (-10 °C) during 15 minutes to stop microbial processes. Initial and final NH_4^+ concentration were determined from filtering 1N KCl extracts from each flask using the phenol hypochlorite method (Solorzano, 1969). KCl extracts were made by adding 40 ml of 2N KCl to 40 ml of incubated sediment slurry and maintaining in dark for 1 hour prior to filtration. Nitrate concentration was also measured colorimetrically with a Seal-3 QuAAtro AQ2 autoanalyzer (Seal Analytical Ltd., Segesworth, UK) in initial and incubated slurries following APHA (2005). Sediment C and N contents were analyzed in an Elemental Analyzer Perkin Elmer Series II 2400. Sediment P was determined by the persulfate oxidation digestion method (Menzel and Corwin, 1965). Dissolved oxygen, conductivity, pH and Eh were measured in the lab before and after slurry incubations to test any change on redox conditions. Gross nitrification rates over the incubation period was measured as the difference in NH_4^+ concentrations between incubations in which nitrification was allowed to occur and those in which nitrification was inhibited with the chemical nitrapyrin. It was assumed that ammonification and heterotrophic NH_4 uptake were uninhibited in both set of flasks. The NH_4 increase in the flasks containing nitrapyrin was a result of inhibited NH_4oxidation.

Nutrient spiraling and field ^{15}N tracer addition

Three short-term enrichments following recommendations of Webster and Erhrman (1996) and Martí and Sabater (2009) were performed by whole-stream *in situ* additions: two NH_4^+ additions (27 May 2013 and 7 June 2013) to determinate uptake length of ammonium, and one $^{15}NH_4$ enrichment (18 June 2013) to trace $^{15}NH_4$ pathways within the stream ecosystem.

Ammonium additions- amount of added NH_4^+ were adjusted to raise by 3 the nutrient concentration in the stream. Stream flow (Q, l/s) were measured at the injection days by stream gauging, using a flow prober (FP101, Global Water). In both additions, we used a solution of 8.5 g/l of NH_4Cl and 350 g/l of NaCl as conservative tracer, dissolved in stream water, which was released at a constant rate of 30 l/h during 1.5 hours using a peristaltic pump. Conductivity was measured every 10 minutes. At time this conservative tracer concentration was constant through time at the downstream end of the study reach (steady state), we collected water samples along the study reach. In the first addition, according to the nutrient spiraling lengths described in other similar studies (e.g. Ensign and Doyle, 2006), we selected a reach segment of 210 m. Collected points were choose to be enough far from the injection point to ensure a complete nutrient spiraling: a background sampled site (10 m upstream from the injection point), and 7 locations downstream, coinciding with the above mentioned characterized stream segments: 1 (25 m), 2 (41 m), 3 (74 m), 4 (115 m), 5 (135 m), 6 (175 m) and 7 (210 m) (Fig. 3.3). This first experiment suggested a longer nutrient spiraling, and the next addition was extended to 1050 m of stream reach adding 4 more downstream sampled sites: 8 (430 m), 9 (636 m), 10 (800 m) and 11 (1050 m). We collected water samples in all the selected locations before the nutrient

injection (baseline samples) and in the plateau steady state (enriched samples). All samples were stored at < 4° C and transported to the lab, where NH_4 concentrations were measured in continuous-flow analyzer (AXFlow AutoAnalyzer 3), following APHA (2005).

To calculated N spiraling metrics, enriched- NH_4^+ concentrations and conductivity values were corrected with the basal measures. The ratio of these variables, NH_4^+ concentration by the conductivity, were used to calculate nutrient spiraling parameters (Newbolt et al., 1981; Stream Solute Workshop, 1990; Martí and Sabater, 2009): retention rate (K_c) was estimated as the slope of the linear regression between de logarithmic (ln) values of this ratio by the stream distance (m), and uptake length (S_w) was calculated as the inverse of K_c. Because uptake length is strongly influenced by discharge, uptake lengths can only be directly compared when discharge is similar, and we eliminated this influence by calculating the uptake velocity coefficient (V_f) (Stream Solute Workshop, 1990; Davis and Minshall, 1999). Uptake velocity (V_f) and uptake rate (U) were measured as:

$$V_f = (h*v) / S_w \qquad \text{(Eq. 3.7)}$$

$$U = C_b*Q/S_w*w \qquad \text{(Eq.3.8)}$$

where h is the mean depth (m), v is the mean flow velocity (m/s), w is the mean channel width (m), Q is the stream flow (l/s) and C_b is the basal nutrient concentration (mg/l). Residence time (min) of NH_4 was calculated with the uptake length (m) and water velocity (m/min). Flux of nitrogen (mg N/s) was estimated with the stream NH_4 concentration (mg N/l) and the stream flow (l/s).

In situ $^{15}NH_4$ tracer addition- the injection solution consists of 159 mg ^{15}N (as 10% ^{15}N enriched $(^{15}NH_4)_2SO_4$, Sigma Aldrich) and 300 g/l of NaCl as conservative solute tracer, over 5.5 h using a fluid metering pump at constant rate of 23 l/h. At the end of the enrichment $\delta^{15}N$ was increased 300 ‰ in the stream water ammonium but in a negligible increase of the total ammonium concentration. Water samples for chemical analysis and $^{15}N\text{-}NO_3$, $^{15}N\text{-}NH_4$, $^{15}N\text{-}N_2$, $^{15}N\text{-}N_2O$ measures were collected upstream of the studied reaches (-5 m) and in 4 downstream locations along the stream reach: 25 m (1), 41 (2), 115 (3) and 175 m (4). We assumed all the solution is completely mixed with stream water at the first sampled station. Since we focus on ammonium processes and transformation that take place in a very short time periods we selected a small length reach in order to minimizing, at much as possible, the loss of ^{15}N tracer. For the same reason, we don't aim to ^{15}N label helophytes, since they may need a longer time to assimilate and show any ^{15}N increase. Conductivity was measured in the last sampled point, every 10 minutes, to control the solution reaches all sampled stations. The experiment was conducted from 11 am and the tracer plateau state was reached at 40 min, therefore all the time was under light condition. Water samples were collected before the isotope injection as baseline values, during the plateau steady, and the day after the enrichment to ensure the system returned to the natural conditions and were stored at <4° C and transported to the lab where were stored frozen. Samples for $^{15}N\text{-}NH_4$ and $^{15}N\text{-}NO_3$ analysis were processed following the diffusion method (Sorenson and Jensen, 1991; Sigman et al., 1997; Holmes et al., 1998). Briefly, 56 g of NaCl, 4.2 g of MgO and a filter packet were added to 1,400 ml of the stream water sample and incubated in a shaker at 38-40 °C for 2 weeks to catch-up $^{15}N\text{-}NH_4$. The filter packet consists of a pre-combusted 1-cm glass-fiber filter (Whatman

GFD) steeped with 25 ml of 2M H_2SO_4 and sealed between two Teflon filters (Millipore white nitec LCWP 25-mm diameter, 10-gm pore size). After this first incubation, filters were removed and placed in a dessicator for two days. 1 g of Devarda´s Allow and a new filter packet were added to the same bottle for a second incubation in order to measure $^{15}N\text{-}NO_3$. Filters were encapsulated in tins, placed in a 96-well titer plate and sent to the stable isotope laboratory at University of California at Davis for ^{15}N: ^{14}N ratio analysis by IRMS. For ^{15}N analysis of dissolved N_2 and N_2O we followed the procedure as described in the headspace method (Hamilton and Ostrom, 2007). At each station, 40 ml of water was collected in 60 ml plastic syringes and all visible air bubbles were expelled. This process was done in a bucket filled of stream water and syringes were kept submerged to avoid air contamination. Replicate samples were collected from each station. After all the samples were collected, needles were affixed to the syringes and 20 ml of high purity helium was incorporated to the syringes. Samples were then shaken gently for 5 mints to allow the equilibration of dissolved gasses between water and He headspace. After that, approximately 20 ml of the headspace gas was injected into pre-evacuated exetainers and shipped to stable isotope laboratory at University of California at Davis for ^{15}N:^{14}N ratio analysis by IRMS-Gas chromatography. Measurements of ^{15}N:^{14}N ratios were expressed as $\delta^{15}N$ values (units of ‰).

Calculations of ^{15}N flux-mass balances and nutrient spiraling metrics followed the methodology described in Mulholland et al. (2000). We computed the total $^{15}NH_4$ flux (mg ^{15}N/s) at each station $_i$ following the equation:

$$^{15}N\text{-}NH_4\ flux_i = (\delta^{15}N\ -NH_{4i}/1000)\ X\ 0.003663\ X\ Q_i\ X\ [NH_4\text{-}N] \quad \text{(Eq. 3.9)}$$

where Q is the stream discharge (l/s) and [NH_4-N] is the stream ammonium concentration. We assumed stable and equal NH_4 and Q for all the stations during the experiment (i.e., 0.04 mg/l and 88 l/s). Finally, we computed tracer $^{15}NH_4$ mass flux (µg/s) at each station i by subtracting baselines values. The total NH_4^+ retention rate (K_c), uptake NH_4^+ length (Sw), uptake rate (U), transfer velocity (V_f) and residence time (min) were calculated as described above, in the nutrient addition method.

N dynamic model

Based on the data obtained from the stream characterization, we built a 4-compartment stream N model that simulated the tracer addition in our stream reach and predicted the ^{15}N values in every compartment over time and distance, following the general scheme proposed by Wollheim et al. (1999). The model includes ammonium and three biotic pools (BOM, HOM and seston) involved in the dissolved N uptake and transformation. Since our tracer experiment did not last long, consumer compartments were not considered in this study. The model assumes that the stream is at steady state with regard to N (inputs equal outputs). The model was partitioned into the four stream segments already defined, and all standing stocks and biomass measurements were converted to mg N/m^2 by segment. Fluxes of ^{15}N into and out of compartments were computed using methods described by Hall et al. (1998), Tank et al. (2000) and Dodds et al. (2000) for nitrogen dynamic

studies in streams. Uptake of dissolved nitrogen into primary uptake compartments (BOM, HOM and seston) was determined by:

$$U_i \text{ (g N /m h)} = {}^{15}\text{N-NH}_4 \text{ flux}_i \text{ X (L/S)} \quad \text{(Eq. 3.10)}$$

where U_i is the uptake rate for each stream segment, ${}^{15}\text{N-NH}_4$ flux_i is the ${}^{15}\text{NH}_4$ concentration entering the segment (g ^{15}N/h), L is the length of the segment (m) and S is the uptake length (m) obtained from the ^{15}N enrichment. Then, we apportioned the uptake rates of ^{15}N to each compartment based on proportional substrate respiration rates using the following formula (Hall et al., 1998):

$$F_{NH4,y} = U_{NH4} \text{ X } (R_y / R_{total}) \quad \text{(Eq. 3.11)}$$

where y is the uptake compartment (BOM, HOM, seston), and R = (respiration rate x standing stock) for each uptake compartment. Respiration rates (mg N/m^2 h) were calculated by the transformation of metabolism incubations to mg N/m^2 h (with C:N ratios) and standing stocks (mg N/m^2) were estimated by the N microbial biomass. The model assumed that the entire N biomass of each uptake compartment was uniformly involved in N cycling (e.g. no separation was made between the rapid-turnover microbial compartment and the non cycling detrital N).

3.4 RESULTS

Stream characterization

Conditions in the Arroyo Grande del Molinillo at the beginning of the experiments were typical for later spring-early summer (Table 3.1). During the experiments, stream discharge was relatively stable, oscillating between 70 and 112 l/s. Vertical hydraulic gradient (GHV) showed low but positive values, suggesting slight, almost negligible upwelling (Table 3.1). Mean permeability values (Kh) were also very low in the entire reach stream, showing higher values in the stream sites dominated by sandy riverbed. Diurnal oxygen pattern indicated a slight increase during the morning with a maximum peak at middle day, followed by a marked decreased in the oxygen concentration (Fig 3.4).

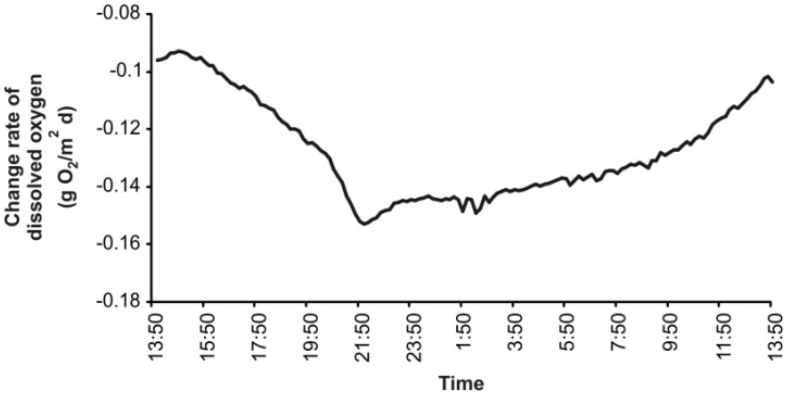

Fig. 3.4: Daily oxygen rate exchange in Arroyo Grande del Molinillo.

During the studied daily cycle, dissolved oxygen change showed negative values (negative NEE_i values), indicating that respiration never was compensated by productivity.

Nutrient concentrations in stream water were relatively high, with NO_3^- controlling the total N pool. Overall, in the study reach, whole stream metabolism showed heterotrophy (P/R <1, Table 3.1). N content in the microbial compartments were relatively constant along the stream reach, being always higher in HOM than BOM (Table 3.2). Microbial biomass represented about 67 % and 78 % of the total benthic and hyporheic organic standing stocks. C:N ratios were similar among the HOM and seston, and substantially lower than the BOM. Results from metabolism incubations showed an homogeneous metabolism along the stream reach (Table 3.2), where respiration rates of seston and BOM showed similar values, while HOM rates showed a significant higher activity (t-test, p<0,01).

Results from sediments incubations showed positive nitrification rates varying 1.67- 5.86 μg $N\text{-}NH_4^+$ /g DW day in the first two segments (Table 3.2). Nitrification rates were negative (-1.09 and -6.48 μg $N\text{-}NH_4^+$ /g DW day) in the last two stream reaches. Control incubations, with added NH_4^+, indicated positive values in all stream segments (4.57, 0.15 and 0.21 μg $N\text{-}NH_4^+$ /g DW day), with the exception of last one (-10.02 μg $N\text{-}NH_4^+$ /g DW day).

Table 3.2: Nitrogen content, C:N molar ratios, gross primary productivity and respiration of the BOM, HOM and seston pools in the four segment reaches of Arroyo Grande del Molinillo: 0-25 m (1), 25-41 m (2), 41-115 (3) 115-175(4).

	1	2	3	4
Nitrification rates				
(µg N/g DW sedim /d)	1.67 (4.57)	5.81 (0.15)	-1.09 (0.21)	-6.48 (-10.02)
N standing stocks				
BOM (µg N/g soil dw)*	21.48	20.93	14.72	11.20
HOM (µg N/g soil dw)*	32.40	33.98	28.21	33.76
Seston (µg N/l)	88.50	88.50	88.50	88.50
C:N ratio				
BOM	4.59	6.33	4.17	4.09
HOM	1.39	1.15	1.96	1.86
Seston	2.72	2.72	2.72	2.72
GPP				
BOM (mg C/m^2/h)	1.76	1.74	0.56	-2.40
Seston (mg C/m^2/h)	0.94	4.19	1.39	3.19
R				
BOM (mg C/m^2/h)	17.14	7.99	9.07	8.30
HOM (mg C/m^2/h)	38.95	50.57	39.59	59.77
Seston (mg C/m^2/h)	12.88	11.18	4.10	9.03

NH_4^+ and $^{15}N\ H_4^+$-tracer *in situ* additions: uptake lengths and rates

During the short and long NH_4^+ additions, the steady state along the studied stream reach was achieved after 40 and 180 minutes of starting additions, respectively (Fig. 3.5). NH_4^+ uptake showed a slightly steeper slope in the long enrichment and, by hence, the uptake length (S_w) was slightly shorter (Table 3.3).

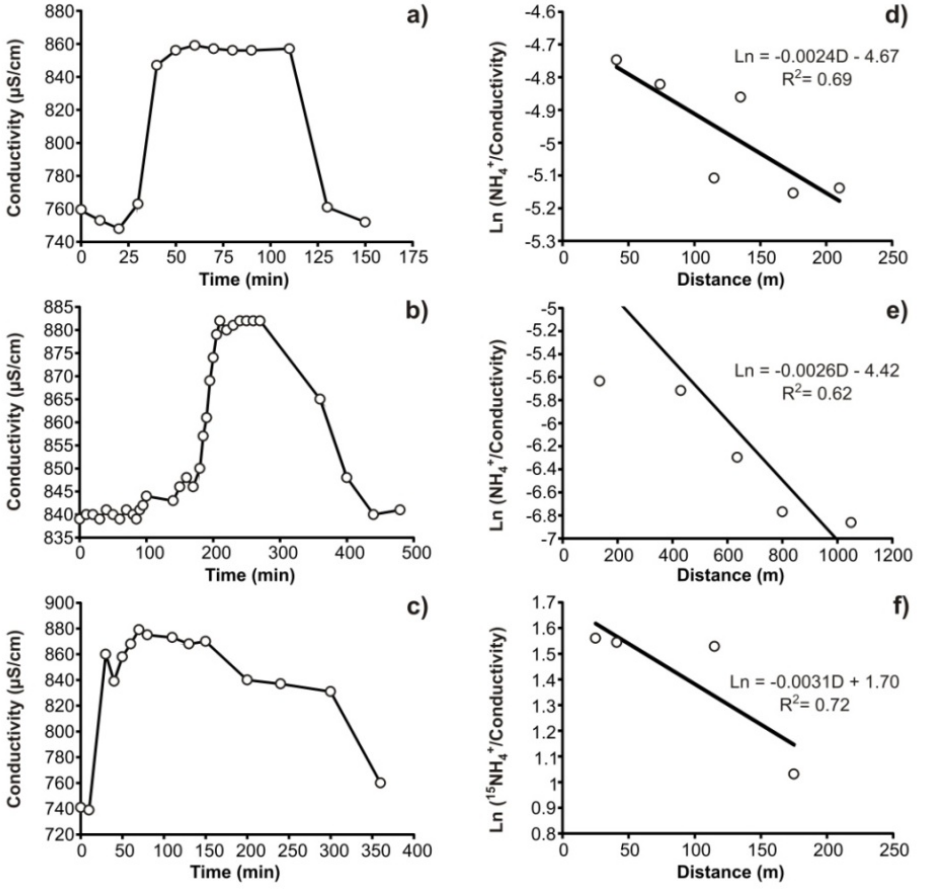

Fig. 3.5: a) b) and c) represent conductivity results in the NH_4 short, NH_4 long and $^{15}NH_4$ enrichments. d), e) and f) represent log-transformed NH_4 versus stream distance from the same experiments.

Uptake rates varied from 140.7 to 182.9 µg N/m^2 min. A higher rate was found in the long enrichment due to stream NH_4^+ concentration which increased from 40 µg/l up to 60 µg/l. Finally, our estimations in Arroyo Grande del Molinillo showed relatively low uptake velocities between 6.9 and 7.48 mm/min, and long residence times of NH_4^+ in the water column of 53.0-57.9 min.

Table 3.3: Uptake lengths and rates for NH_4 based on the three addition experiments.

	S_w (m)	U ($\mu g\ N/m^2\ min$)	V_f (mm/min)	K_c (m^{-1})	Residence time (min)
NH_4 enrichment					
Short	417	140.69	6.91	0.0024	57.9
Long	385	182.86	7.48	0.0026	53.0
$^{15}NH_4$ enrichment	333	140.80	8.65	0.0030	46.0

During the $^{15}NH_4$ tracer addition, conductivity values showed that the steady state was reached at 35 min (Fig 3.5). Isotopic measurements reported baseline values of $\delta^{15}NH_4$ and $\delta^{15}NO_3$ in ~12‰ and ~3‰, respectively. Spatial patterns of $\delta^{15}NH_4$ enrichments reflected longitudinal abundances, with $\Delta\delta^{15}N$ up to 380‰ in the first 115 m, declining rapidly as distance increased (Fig 3.6a). ^{15}N-labeled ammonium showed no enrichment in the samples taken the day after the enrichment, indicating the temporariness of the tracer addition (Fig 3.6a). $\delta^{15}NO_3$ during the addition showed a slightly enrichment above baseline values, being more marked at the end of the reach, with a maximum peak of 30 ‰ at 175 m (Fig 3.6a). $\Delta\delta^{15}NO_3$ after the ^{15}N addition was negligible and $\delta^{15}NO_3$ resulted homogeneous along the stream reach. $\delta^{15}N_2$ and $\delta^{15}N_2O$ values were extremely low and $\Delta^{15}N$ were negligible along all the stream reach (Fig.3.6b). Headspace N_2 mass concentrations were 6 to 10 times more than expected values suggesting possible contamination of vials.

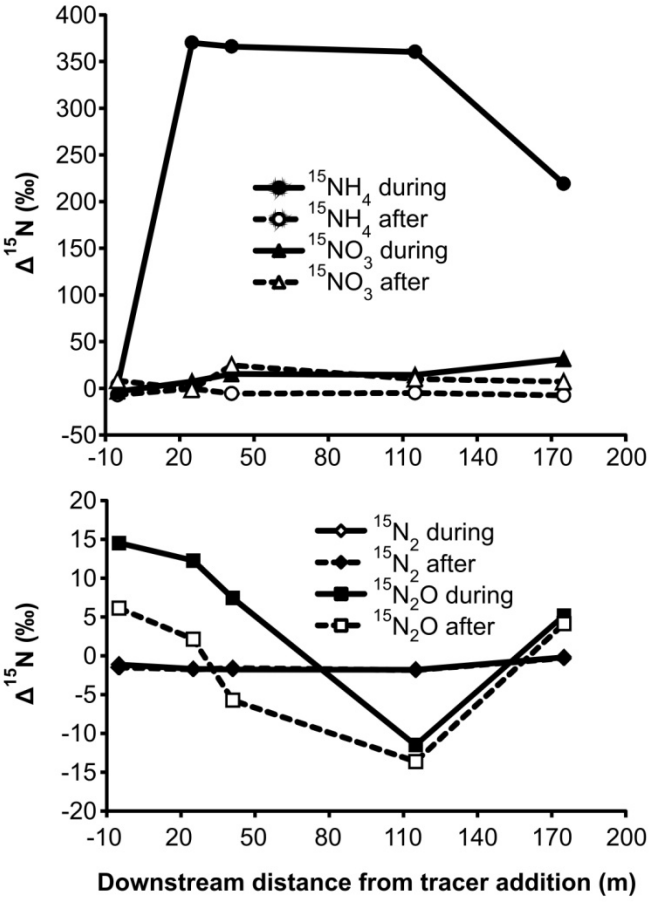

Fig. 3.6: Longitudinal profiles of observed $\Delta\delta^{15}N$ labels of NH_4 and NO_3 in the study reach, the day of the tracer release at times 1.5 and 22 hours after the isotope injection. Increments (Δ) were calculated as $\delta^{15}N$ of each sample minus the baseline value.

Ammonium uptake length was computed from the decline in semi-log plots of $^{15}NH_4$ flux over the stream reach (Fig. 3.5). Whole-stream uptake length was 333 m, somewhat slightly shorter than results from the previous nutrient addition experiments (Table 3.3). As the NH_4 stream concentration at this day was 40 µg/l, the uptake rate per unit area was estimated to be slightly

lower than the other NH_4 additions (140.8 µg N/ m^2 min). Uptake velocity (V_f) and residence time of ammonium in stream were calculated to be 8.65 mm/min and 46 min, respectively.

Compartmental stream model

Respiration rates and standing stocks for the modeled stream compartments, and resulting fluxes are summarized in Table 3.4. Fluxes of $^{15}NH_4$ (mg ^{15}N/h) in the water column showed a slower decreased in the first 115 m, being more marked at the end of the stream reach (Table 3.4). ^{15}N Uptake rates, calculated for each stream segment, were almost uniform, representing a small portion of total ^{15}N fluxes presented in the water column (Table 3.4). ^{15}N fluxes and uptake rates indicated that in the first 115 m the major fraction of the $^{15}NH_4$ in the water column (> 95 %) was fluxed downstream to the next segment, whereas only a small portion was retained into the stream reach. The greatest part of this contained ^{15}N was uptake by the HOM, whereas BOM and seston showed marginal ^{15}N retention rates (Table 3.4). Between the distances 115 m and 175 m, uptake rate and compartmental fluxes showed lower values as a consequence of the decline of $^{15}NH_4$ in the water column.

Table 3.4: Metabolism, standing stocks and N-NH_4 and ^{15}N-NH_4 flux in the modeled pools of BOM, HOM and seston in the four reaches of Arroyo Grande del Molinillo: 0-25 m (1), 25-41 m (2), 41-115 m (3) 115-175 m (4).

	1	2	3	4
Standing stocks (µg N /m^2)				
BOM	0.12	0.15	0.10	0.07
HOM	0.55	0.71	0.60	0.58
Seston	0.04	0.04	0.05	0.05
Respiration (mgN/m^2/h)				
BOM	5.64	1.76	2.54	2.36
HOM	27.99	47.04	22.06	34.09
Seston	4.74	4.12	1.51	3.33
Whole-stream				
Flux $_{NH4}$ (mg ^{15}N/h)	16.92	16.86	16.60	10.10
U (µg ^{15}N /m h)	46.75	50.64	49.86	30.33
% retention*	0.28	0.30	0.30	0.30
% lost dowstream	99.60	98.40	61.00	0,77 [1]
Flux of $^{15}NH_4$ by compartiments (µg ^{15}N /m h)				
Flux $_{NH4\text{-}BOM}$	1.95	0.40	0.94	0.25
Flux $_{NH4\text{-}HOM}$	44.25	50.00	48.65	29.81
Flux $_{NH4\text{-}SESTON}$	0.54	0.25	0.27	0.27
% BOM	4.17	0.78	1.88	0.83
% HOM	94.67	98.73	97.57	98.29
% seston	1.16	0.49	0.55	0.89

3.5 DISCUSSION

Ammonium uptake length and uptake rate

Transformation and assimilation of nitrogen, and specifically of ammonium, on stream ecosystems are complex because involve many different processes resulting from the interaction of hydrologic, chemical, and biological variables (Mulholland et al., 2000, Peterson et al., 2001; Hall and Tank, 2003). Differences in uptake lengths (S_w) may reflect divergences in physical properties within streams, but also differences in biotic nutrient demand and N processing (Newbolt et al., 1981; Ensign and Doyle, 2006). Both additions,

NH_4 and $^{15}NH_4$, showed similar uptake lengths, being slightly higher in the $^{15}NH_4$ enrichment, and within the range of values reported for streams (32 to 900 m; Martí and Sabater, 1996; Peterson et al., 1997; Hall et al., 1998; Mulholland et al., 2000; Merrian et al., 2002; Hall and Tank, 2003). Whole stream uptake rate per unit area (U) and relative NH_4 demand (V_f) values were similar than reported in ^{15}N enrichment in agriculture and urban stream (Arango et al., 2008). Our third-order stream Arroyo Grande del Molinillo under the influence of agriculture showed a relatively long ammonium uptake length (> 333) and high residence times (> 46 m). In the opposite way, Mulholland et al. (2000) reported short uptake lengths (23–27 m) and low residence time (5 min) in a first-order deciduous forest stream in eastern Tennessee (the Walker Branch). Several studies have shown spatial and temporal variation in S_w among and within streams, which have been largely related to temperature and hydrological factors (Aumen et al., 1990; D'Angelo and Webster, 1991; Martí and Sabater, 1996). Despite this trouble comparing S_W values among different streams, in a broad meaning, long processing lengths reflect low rates of material cycling. Consequently, our results on Arroyo Grande del Molinillo suggested that streams influenced by agriculture are systems poorly efficient in the retention of ammonium, contrary to forest streams which shows a tight cycling and high biological demands (Newbold et al., 1981, 1982; Elwood et al., 1983; Peterson et al., 2000; Ensign and Doyle, 2006). However, uptake rates differ considerably in both contrasting systems: the assimilation by the biota in our agriculture stream was until 7 times upper than those recorded in the pristine forest stream (141-183 *vs.* 22-37 μg N/m^2 min). NH_4 uptake rate has been shown to be strongly dependent on concentration in a single stream when is a limiting nutrient (Mulholland et al., 2000). Our study suggests that this concentration

dependence of the uptake rate is expected to be found among streams independent of its environmental settings. According to this, NH_4 uptake length represents a measure of nutrient uptake efficiency (uptake/supply) rather than uptake rate *per se*, as suggested by others (Mulholland et al., 2000). Whether ammonium is a limiting nutrient or is in excess and the influence of biological demands on nutrient uptake cannot be determined by these rate processes.

Land use influence on the transformation of ammonium and saturation of nitrification process

The added $^{15}NH_4$ served as an ammonium tracer and followed pathways identical to those followed by ammonium entering the stream from natural sources. Measured fluxes of $^{15}NH_4$ in the water column along the stream reach, indicated a slow and small $^{15}NH_4$ decrease in the first 115 meters, where more than 95% of the ammonium is exported downstream.

Nitrogen uptake related to the biological communities of the benthos, hyporheic zone and seston accounted for only a small portion of the total ammonium in the water column. Among these biotic N pools, the microbial activity associated to the hyphoreic zone appeared to be largely responsible for the N uptake in the stream system. The importance of the hyporheic zone on the N processing has been largely recognized (Findlay, 1995, Boulton et al., 1998). The heterogeneity of the habitat, with biologically and chemically disparate microzones to co-occur, and the ability to provide stable refuge for stream organisms have been cited has the main factors conditioning the high efficiency of the hyporheic zone on nitrogen dynamics (Orghidan, 1959;

Boulton et al., 1998). Discharge and direction (i.e. downwelling/upwelling) are the main variables controlling the intensity and type of nutrient transformation in the hyporheic zone (Boulton et al., 1998; Storey et al., 2004). Our hydraulic data indicates that Arroyo del Molinillo stream did not receive substantial groundwater discharges currently but subsurface flow can be supplied the high biological demands observed.

Although the magnitude of the nitrogen uptake assessed could be underestimated and DIN processed in these compartments may be greater downstream close to the spiraling length, biological demands of ammonium in this stream are rather low. In streams with relatively low nitrogen inputs, higher concentrations of nitrate compared to the low concentrations of nitrite and ammonia indicated nitrification was a major controlling process (Tzoraki et al., 2007). During the ^{15}N enrichment experiment in Arroyo Grande del Molinillo observed increases in $\delta^{15}NO_3$ pool were extremely low, compared to other tracer studies (e.g. Mulholland et al., 2004), suggesting appreciable, but very low nitrification rates. Clearly, the assessment of ammonium uptake and retention depends on understanding the mechanisms driving nutrient uptake (O'Brien et al., 2007). The rate of nitrification is controlled primarily by the availability of ammonia which was not a limiting factor in our studied stream according our nitrification experiments. On the other hand, nitrification rate in streams generally increase as ammonium concentration increase (Peterson et al., 2001; Kemp and Dodds, 2002). Our results suggest a minor importance of nitrification pathway in streams with high ammonium loads than reported in streams with lower NH_4^+ concentrations (2-4 µg/l; Davidson et al., 1991, Stark and Hart, 1997; Mulholland et al., 2000). This could be related to the nonlinear response of nitrification when a threshold in the ammonium concentration is exceed (Dodds et al., 2010). This behavior

has been reported by Kemp and Dodds (2002) through sediments incubation experiments, which observed that nitrification rates reached a significant plateau state at NH_4 concentrations above 30 μg/l. Similar NH_4 concentration were found in Arroyo Grande del Molinillo that could explain the low observed nitrification rates. Furthermore, NH_4 demands via nitrification in streams influenced by urban and agriculture practices have been showed to display saturation of biological demands, indicating decreased uptake efficiency at NH_4 concentration in a similar order of magnitude that above mentioned (Arango et al., 2008, Dodds et al., 2010). Once the threshold is crossed, it is assumed that return will be difficult because ecosystem responds by assuming a new stable state (Bellwood et al., 2004; Dodds et al., 2010).

High N_2 mass values measured and low $\delta^{15}N_2$‰ values during the ^{15}N addition lead us to think of inadvertent contamination by atmospheric N_2 introduced during sampling or storage before measuring (as noted in other studies; Tobias et al., 2001). Since air contamination should be unimportant in the case of N_2O, $\delta^{15}N_2O$ changes may corresponded to a poor analytical precision or natural variations, rather than as consequence of the ^{15}N addition (Tobias et at., 2001). During the ^{15}N enrichment, the dilution of the ^{15}N label over the NO_3 pool before reaching N_2O and N_2 compartments increased the difficulty of the registering significant $\Delta\delta^{15}N_2O$ and $\Delta\delta^{15}N_2$. Great NO_3 concentrations, generally, increase denitrification rates in streams (Bernot and Dodds, 2005). Although we could not tracer the denitrification pathway through the addition of $^{15}NH_4$, NO_3 concentrations in Arroyo Grande del Molinillo were enough high, but below the saturation limits (Arango et al., 2008), to expect this process to play an import role in the N cycle of this stream.

Our study provided evidences that streams draining agriculture lands in semi-arid regions are receiving high NO_3 loads and can be partially saturated in NH_4 concentration. Mulholland et al., 2008 that the efficiency of biotic uptake and denitrification declines as nitrate concentration increases, reducing the proportion of in-stream nitrate that is removed from transport. Our results suggest that also ammonium uptake and nitrification follow the same pattern. In agricultural streams, nitrification can be saturated by elevated N loadings, increasing the toxicity of ammonia. The hyporheic zone display a key role on nitrogen metabolism and need to be considered in restoration programs of semi-arid streams in order to provide subsurface flow increasing the total nitrification and decreasing evapotranspiration losses. Our study provide a baseline from which to examine further the NH_4 dynamics in agriculture (NO_3-enriched) semi-arid streams. The resilience of ammonium transformation pathways need to be explored in streams to make mechanistic predictions of thresholds under environmental conditions, including disturbances.

ACKNOWLEDGEMENTS

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CHAPTER 4

Nitrogen dynamics in an oligotrophic lake through an ecosystem-scale isotope tracer experiment

"The value of lakes, their immense scientific interest, their importance as threatened habitats and their relevance to the human community as resource, is so overwhelmingly obvious as to need no further amplification"

O´Sullivan and Reynolds

4.1 SUMMARY

Lakes have a crucial role as regulators, controlling the transport and transformation of nitrogen compounds. A full comprehension of these processes in unimpacted lakes provides the baseline by which we can assess the consequences of human perturbation in these aquatic ecosystems. In this study, we performed a whole-scale short term $(^{15}NH_4)_2SO_4$ addition in an oligotrophic lake (Somolinos Lake) to trace the main ammonium dynamics. During 3 weeks after the injection, ^{15}N label was traced through the main compartments regarding the N cycle: DIN compounds, submerged macrophytes and sediments. Overall, results showed short ammonium residence time, where much of this N was rapidly lost through downstream fluxing. However, submerged macrophytes exhibited a moderate rapid uptake of ammonium (in a magnitude of days), suggesting that in Somolinos lake, these dense meadows act as main mechanisms retaining the dissolved nitrogen of the water column. Our study also evidences that the nitrification processes are occurring in the system, converting rapidly the ammonium into nitrate and making nitrogen available for denitrification.

Key words: isotope enrichment, oligotrophic lake, lake-uptake rate, submerged macrophytes,

4.2 INTRODUCTION

Landscape features, such lakes, have an important role as regulator of transport and transformations of Nitrogen within the global N cycle (Kling et al., 2000; Francis et al., 2007). Nutrient inputs from human activities, even in low level, influence the function and the structure of lake ecosystems, especially in oligotrophic lakes (Hadden and Bunn, 2005), modifying numerous ecosystem services that they provide (e.g. biomass production, recreation, wild life habitat, biodiversity, among others; Rodríguez et al., 2006). Depending on the turnover times, N become transformed to other N compounds, stored in biota, settled for long time or exported out to the system (Epstein et al., 2012). Further understood of the processes controlling nitrogen uptake and retention in unimpacted lakes, provide the baselines by which we can assess the consequences of human perturbations within these ecosystems.

Denitrification, sedimentation and uptake by plants are the main mechanisms of N retention In lakes (Saunders et al., 2001). Indeed, denitrification has been recognized as one of the most important mechanism for N removal in aquatic systems, decreasing the total N transported to the oceans (Seitzinger et al., 2006). Generally, denitrification can occur in any system where nitrate and organic matter are available (Seitzinger et al., 2006), increasing the rate with greater NO_3 concentrations in the water (Piña-Ochoa et al., 2007). Some oligotrophic lakes have showed lacks in the organic carbon production necessary to drive effective sedimentary denitrification, making these systems more vulnerable to counteracted NO_3 terrestrial inputs (Finlay et al., 2007). In these N-limited systems, we could also expected a high relative importance of the couple nitrification-denitrification (Seitzinger, 1988; Piña-Ochoa et al., 2007; Vila Costa et al., 2014). The relevance of the nitrification

on the N cycle has been widely reported (Ward, 2011). Despite nitrification can be considered a well-known process, measurements of rates and variables controlling are relatively rare in lake ecosystems, especially in the water column (Small et al., 2014). The relative importance of nitrification/denitrification on the nitrogen dynamics according several environmental constrains needs to be revisited using novel methodologies of water bodies.

Submerged macrophytes can also play a central role in nutrient cycling, especially in small, shallow lakes (Ozimej et al., 1993, Scheefer, 1998), contributing through uptake to in-lake nutrient transformation and inmobilization (Marion and Paillisson 2003; Vermeer et al., 2003). Indeed, besides the role of sediments as main reservoir of N, aquatic vegetation is critical in temporal retention of nutrients, that otherwise be lost from the lake food web (Barko et al., 1991; Tobias et al., 2003; Epstein et al., 2012). Evidences reflect that macrophyte species can obtain N from their shoots, water via, and from the sediment through their roots; however the question regarding on the relative importance of each source and their ecological implications is being a subject of controversy for many years (Sculthorpe 1967; Zhang et al.; Barko and Smart, 1980; Ozimek et al., 1993; Eugelink 1998; Madsen and Cedergreen 2002; Hasegawa et al. 2005). Studies examining N uptake of submerged macrophytes using field-based research, particularly stable isotope approaches, are scarce, and more research focusing on the role of macrophytes as source or sink of N are needed (Rodrigo et al., 2006).

In lakes, most of the studies concerning N dynamics and biogeochemical processes, such nitrification-denitrification, have been carried out based on microcosms approaches (Mulholland et al., 2004), mass balances of N budgets

(Piña-Ochoa and Álvarez-Cobelas, 2006) or nutrient additions (Arp et al., 2006). Methodological and logistical restrictions have limited our understanding of interactions between various lake compartments and, by hence, the functioning of these ecosystems as a whole (e.g., the difficulty to deal with the large spatial and temporal heterogeneity of N_2 production in aquatic ecosystems, Piña-Ochoa and Álvarez-Cobelas, 2006). The use of whole-ecosystem isotope enrichment approach provides an ideal way to examine flows through multiple pools simultaneously while maintaining natural hydrologic and biogeochemical gradients (Peterson and Fry, 1987; Tobias et al. 2003). In the last decades, several studies have used deliberate ^{15}N additions to trace nitrogen flows in freshwaters (e.g., Kling, 1994; Peterson et al., 1997; Hamilton et al., 2001; Webster et al., 2003). In lakes, although whole ecosystem ^{15}N enrichments are scarce, this approach have lent insights into the relative importance of pelagic and benthic producers, spatial migration of consumers and biogeochemical transformations of N (e.g., Kling et al., 1994; Hadwen et al., 2005; Epstein et al., 2012; Armengol et al., 2012).

In this study we performed a whole-system $^{15}NH_4$ addition to explore the transformation, transport and removal of ammonium through an oligotrohic lake. We enriched the DIN pool with ammonium (NH_4) since lower NH_4 concentrations in Somolinos lake provided easier and faster ^{15}N enrichment in the entire system; moreover, the small size of this lake made is as tractable unit to study whole ecosystem dynamics. Our main goal was to study the ammonium uptake, retention and transformation trough the entire lentic ecosystem, focusing on macrophyte species to account for the total N fraction retained in this biota compartment, discerning the relative importance of the two main N sources, sedimentary and in the water column, as the preference by macrophytes uptake. Finally, the tiny processes of nitrification-denitrification

are evaluated as it may account for a large fraction of the ammonium transformation in the lake.

4.3 MATERIAL AND METHODS

Study site

The study was performed in Laguna de Somolinos, a permanent karstic shallow lake (8 m depth) located in the Tajo River Basin, Spain (41°15´12´´N 3° 3´51´´W, Fig. 4.1). Somolinos lake is located at 1250 m of altitude, in an area with 538 mm of annual precipitations and mean daily temperatures ranging from 15.3°C in summer to 6.5°C in winter (Table 4.1). The lake has a well defined water inflow and outflow through Bornova stream which is feed by the groundwater discharges of a karstic aquifer (Almazán-Sur system 02.16). Also the lake receives groundwater discharges from this aquifer by means different springs located at the bottom lake. Somolinos is a well-mixed lake during the entire year. The lake is oligotrophic (mean Chl-*a* concentration of 1.8 µg/l) and oligohaline (conductivity of 345 µs/cm). Somolinos holding a high number of rare and endangered vegetal and animal species, which promote their environmental protection and limit its recreational use (Natural Park of Sierra de la Pela y Laguna de Somolinos, Real Decreto 161/2002). *Chara hispida, Chara vulgaris, Groenlandia densa*, *Sparganium emersun* were the most abundant submerged macrophytes growing in the Lake. The littoral zone was dominated by helophytes (*Cladium mariscus, Juncus inflexus and Phragmites australis*) and by poplars (*Populus nigra*) and willows (*Salix neotricha*). Extensive stands of *Carex riparia*, *C. elata* and *C. paniculata* are located in flooded areas out from the lake to the North and Southstands. *Ranunculus*

trichophyllus appeared in Bornova stream although its cover is almost negligible to ecosystem-scale.

Lake characterization

Three sampling points were used to characterize the lake: upstream the lake (inflows) at the Bornova stream, downstream the lake (outflows), and at the center of the lake in the maximum depth location (Fig 4.1). The tracer addition experiment was performed from June 18 to 9 July 2013. A further ecosystem characterization was performed during the April and May 2013.

Hydrodynamics, water quality and nutrient dynamics- To build the daily water balance of the lake, inflows and outflows were measured 4-times/day during tracer experiment and weekly during the rest of the year using a FP101 flow probe (Global Water). Water level was recorded every 6 h using a pressured transducer Levelogger 3001 (Solinst) submerged in the lake center, which was barometrically compensated (barologger, Solinst). Water volume was calculated based on a bathymetric assessment of the lake carried out 2 months before the study by using a Furuno FE-4300 echo-sounder. In addition, on June 5th, water flows and residence times were measured by the injection of Rhodamine WT. Meteorological variables were recording through a Campbell BWS200 automatic portable station, including a ARG100 tipping bucket raingauge (Young) and a CS-300 pyranometer (Apogee), used to compute evapotranspiration following a modified Penman equation (Shuttleworth, 1993).

During tracer addition period, dissolved oxygen, temperature, pH and conductivity were measured twice per day at the upstream and downstream

sampling stations using a proODO (YSI) and a Crison MM40 portables probes. In situ water column profiles of conductivity, temperature, pH and dissolved oxygen were recorded also twice per day with a CTD SeaBird-19 (Seabird Electronics Inc). Transparency on the lake was measured daily using a Secchi disk

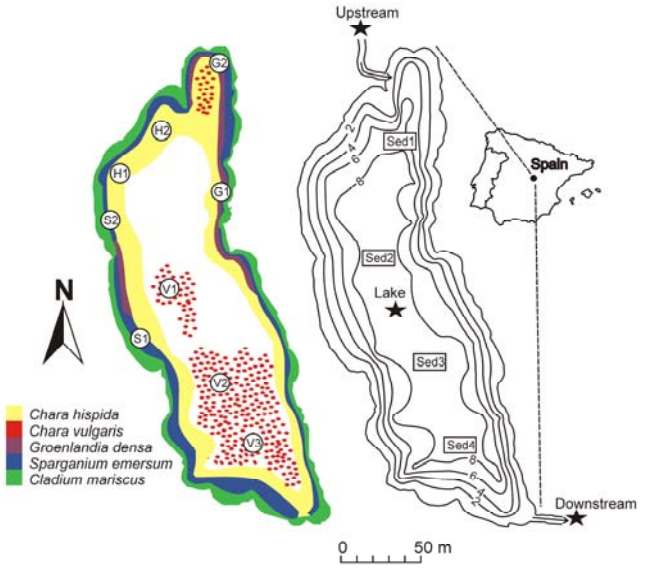

Fig. 4.1: Location map and schematic diagram of the lake Somolinos. Surfaces occupied by each submerged macrophytes species: *Groenlandia emersun* (676 m^2), *Chara vulgaris* (441 m^2), *Chara hispida* (5,550 m^2), *Sparganium emersun* (2,587 m^2).

While stream samples were gathered manually, lake samples were collected with a 5-L van Dorn bottle. Ultraclean PVC bottles were used to store water samples at 4 ºC until analysis, which was conducted within two days after sampling. Samples for organic C were stored in glass vials. Nitrate, nitrite, ammonia, total nitrogen, soluble reactive phosphorus (SRP) and total phosphorus were measured colorimetrically with a Seal-3 QuAAtro AQ2 autoanalyzer (Seal Analytical Ltd). Total organic carbon (TOC) and dissolved organic carbon (DOC) were measured with a Shimadzu TOC-VCSH

(Shimadzu Corporation, Kyoto, Japan) analyzer following the methods of APHA (2005). Chl-*a* was measured spectrophotometrically after extraction with methanol (Marker et al., 1980). Gross and net nutrient sedimentation rates were measured monthly from June to August 2013 using sediment traps. Traps consisted on PVC cylinders (32 cm x 2.5 cm; following Bloesch, 1996) which were submerged at 1m and 7 m by pairs in 3 sampling stations (Fig. 4.1) after filled with distilled water during 25-27 days. No preservatives were added when deploying traps, which were maintained < 4º C during field transport. In the laboratory samples were analyzed by total solids (110º C), mineral matter, organic matter (550 °C ignition losses), total organic carbon and total nitrogen (AXFlow AutoAnalyzer 3 and Shimadzu TOC-V).

Vegetation cover and areal biomass- Surveys were done by scuba divers along each of the 3 longitudinal and 8 transversal transects throughout the lake. 625 cm^2 quadrants were used to sampling areal biomass of each submerged plants. 10-12 samples per species were taken to assess biomass which is expressed as dry weight after dried in oven at 85º C during 48 h.

Metabolisms- Phytoplankton, macrophytes and sediment metabolisms were measured by light/dark bottle method during 18, 19, 20 and 21 June 2013. Phytoplankton incubations were done to 1 m depth. Three samples of each *Chara hispida, Chara vulgaris*, *Groenlandia densa* and *Sparganium emersun* were collected randomly and incubated per triplicated at 3 m depth. Four samples of sediments were collected randomly along the lake using a Ponar type sediment grab and incubated by triplicated at 3 m depth. Changes in oxygen concentration were measured by a multisonde HQ40D with a LDO sensor (HACH-LANGE). Incubations were maintained during approximately 4 hours from 11 h am to 17 pm. Net and gross primary production (NPP, GPP)

and respiration (R) were estimated as described in the chapter 3 (Eqs. 3.4, 3.5 and 3.6). After the incubations, plant and sediment dry weights were measured in all incubated samples. Areal metabolism rates were estimated using biomass and bulk density of the first 20 cm of the sediment layer.

Field $^{15}NH_4$ tracer addition, sampling and analysis

The injection solution consists of 7.445 kg of $(^{15}NH_4)_2SO_4$ (157 g of ^{15}N as 10% ^{15}N enriched, Sigma Aldrich) and Rhodamine WT as conservative solute tracer, dripped 2 m upstream the inlet of the Bornova stream into the lake, by gravity, over 4 h. At the end of the tracer addition $\delta^{15}N$ was increased > 2,000 ‰ in the lake water ammonium but with negligible change in the total ammonium concentration. Water samples for chemical analysis and ^{15}N-NO3, ^{15}N-NH_4, ^{15}N-N_2, ^{15}N-N_2O measures were collected at the lake center and up and downstream. Samples were collected before the isotope injection as baseline values, and 12, 24, 36, 48, 60 hours, 1 and 3 weeks after starting the tracer release. Samples were stored at < 4° C and transported to the lab where were stored frozen. In the lab, prior to isotopic analysis, water samples were filtered through Whatman GF/F filters and processed for dissolved inorganic N (^{15}N-NH_4 and ^{15}N-NO_3) through the NH_4 diffusion method (Sorenson and Jensen, 1991). Dissolved N gasses (^{15}N-N_2 and ^{15}N-N_2O) were extracted using the headspace method (Hamilton and Ostrom, 2007). Both methods were fully described in the chapter 3.

Sediment samples (0-10 cm) were collected with a sediment grab in both upstream and downstream sampling points and along a longitudinal transect within the lake (SED1, SED2, SED3, SED4; Fig.4.1). Plant samples consisted on the most abundant species: helophyte samples (*Cladium mariscus* and

Phragmites australis) consisted on green leafs and were taken randomly in different stands along the littoral, submerged macrophytes (*Chara hispida, Chara vulgaris*, *Groenlandia densa* and *Sparganium emersun)* were collected from several sites throughout the lake in order to account spatial heterogeneity on ^{15}N uptake (Fig. 4.1). Since we expected slower ^{15}N enrichment in plant and sediment compared with water ones, we only collected them before the tracer addition (baseline values) and 48 h, 1 and 3 weeks after the ^{15}N release. All samples were promptly stored on ice while being transported to the laboratory where were processed following protocols by Lewis et al. (2001), Parkyn et al. (2001), O'Reilly and Hecky (2002) and Demopoulos (2004). Briefly, sediment and plant were washed with HCl (1M and 5% respectively) during 24 h, or until we observed all carbonates were removed. Finally, samples were rinsed with distilled water and dried in an oven at 110° C (sediments) and 65° C (vegetation, to avoid the loss of volatile organic compounds) during 48–72 h. Dried samples were ground to fine powder with a mortar and 2 mg were stored dry in clean eppendorfs until being analyzed.. Isotopic analyses were performed by both the Stable Isotope Laboratory of the University of Arizona (solid samples) and the University of Californa at Davis (water and gas samples) for ^{15}N:^{14}N ratio analysis by mass spectrometry. Measurements of ^{15}N:^{14}N ratios were expressed as $\delta^{15}N$ values (units of ‰).

Flux of the ^{15}N tracer was assessedby change in $\delta^{15}N$ of samples above baselines ($\Delta\delta^{15}N$). Amounts of ^{15}N in the dissolved nitrogen pools (NH_4, NO_3, N_2 and N_2O) pools, expressed as (mg $^{15}N/m^3$), were calculated as:

$$^{15}N_{DINi} = (\delta^{15}N_{DINi} /1000) \text{ X } 0.003663 \text{ X } TN_{DINi} \qquad \text{(Eq. 4.1)}$$

where i represented the different four N pools, and TN_{DINi} is the N concentrations (mg /l). Total ^{15}N amounts (g ^{15}N) were calculated by

multiplying $^{15}N_{DINi}$ by the total lake volume (m^3). We assumed an equal homogenization and ^{15}N enrichment along the whole lake in samples used for calculations. An estimation of the nitrification rate (g N/ h) was determined over the first 36 and 172 hours after the enrichment following next equation:

$$\text{Nitrification rate} = (^{15}N_{NO3,time}) / (^{15}N_{NH4,\ time} X \text{ time}) \quad \text{(Eq. 4.2)}$$

where time is 36 and 172, respectively. ^{15}N mass associated with each macrophyte sample (g ^{15}N /m^2) was determined from the background-corrected $\delta^{15}N$ values ($\delta^{15}N_{macrophyte}$) and the total N standing stocks (g N/ m^2) for each compartment as follows:

$$^{15}N_{macrophyte} = (\delta^{15}N /1000)\ X\ 0.003663\ X\ (TN) \quad \text{(Eq. 4.3)}$$

where 0.003663 is the ratio of ^{15}N to ^{14}N in air and TN (g N/m^2) is the total nitrogen in each compartment, calculated by multiplying the total biomass and the total N content in %. Rates of ammonium uptake (mg N /m^2 day) by each of the macrophyte species were calculated from the tracer ^{15}N values over the first three weeks after the ^{15}N addition as:

$$\text{Ammonium uptake rate} = (^{15}N_{macrophytes}) / (^{15}N_{water} X \text{ days}) \quad \text{(Eq. 4.4)}$$

where $^{15}N_{macrophytes}$ was calculated from Eq. 4.3 and $^{15}N_{water}$ is ^{15}N:^{14}N ratios in lake water NH_4 and was computed as (^{15}N-NH_4/1000) X 0.003663. Ammonium uptake rates were calculated for collected samples in the first and third weeks, and we assumed that the mean value represented the total uptake rates during the experiment. Nitrogen turnover rates and times for specific macrophytes were calculated using de decline in $^{15}N_{macrophytes}$ values over the three sampled weeks. The slope of the relationship between ln ($\delta^{15}N_{macrophytes}$) and time represents the compartment-specific ^{15}N turnover rates (d^{-1}) (Tank et al., 2000b). This estimation assumes that there is no re-uptake of ^{15}N released

into the water column. The inverse of the uptake rates represented the turnover times (d). Finally, at the end of the first week, we estimated a whole-ecosystem ^{15}N mass balance, summarizing the ^{15}N uptake, storage, transfer and export via downstream. To this end, we calculated how much of the ^{15}N dripped upstream was retained in each of the sampled compartment.

4.4 RESULTS

Hydrodynamics and limnological characterization

Based on the Rhodamine WT temporal and spatial distributions (Fig. 4.2), the residence time was calculated to be 18.4 days, with a hydraulic load of 14.1 m/day. During the study period water outflows were higher than inflows (16,323 and 18,485 m^3/d, respectively) suggesting an important groundwater discharge directly on the lake.

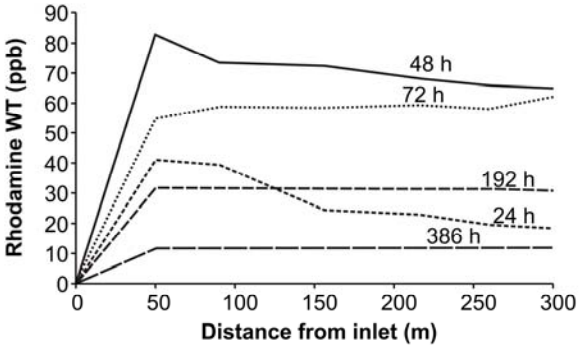

Fig. 4.2: Rhodamina temporal and spatial distribution over the Lake Somolinos after a discrete addition during the experiment. Times are expressed in hours.

The day of addition the water was very clear, with a Secchi depth of 7m, and a homogeneous temperature in the entire water column, with a slight decrease in the deepest zones (Fig. 4.3a). Conductivity profile indicated mixing at all depths, suggesting that the quality of groundwater inputs was very similar to the lake water (Fig. 4.3b). Oxygen concentrations were relatively high in all the water body, with values above 8.5 mg/l, peaking at 3-4 meters depth (Fig. 4.3c).

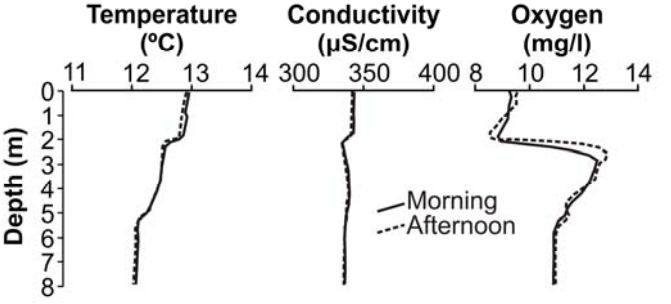

Fig. 4.3: Profiles of (a) temperature, (b) conductivity and (c) oxygen in Somolinos Lakes. CTD data were from two measures (morning and afternoon), the day 18 of June 2013.

Nutrient concentrations were low, with mean NH_4 values of 0.034 mg/l and NO_3 values of 1.5 mg/l, being the main inorganic nitrogen pool, and Chlorophyll-*a* around 1.8 µg/l (Table 4.1). During summer of 2013, mean N sedimentation rates measured was of 0.04±0.01 g N/m^2 day. Table 4.1 shown metabolism results from incubations on sediment and submerged macrophytes. The highest net production rate was exhibited by *Groenlandia densa* whereas *Sparganium emersum* showed lowest values. Respiration of the first 20 cm of sediment resulted in a largest consumption of oxygen but gross production of phytoplankton in the lake resulted almost negligible.

Table 4.1: Physical and chemical conditions and metabolism characterization in Somolinos Lake during ^{15}N tracer addition experiment. (na) not analyzed.

	Parameter	Values	
Physical	Altitude (m)	1,250	
	Surface (ha)	1.9	
	Perimeter (m)	706	
	Max depth (m)	8	
	Volume (m^3)	165,480	
	Residence time	14.1	
	Inflow (m^3 /d)	12,208	
	Outflow (m^3 /d)	18,706	
	Average Precipitation (mm)	538	
	Average Temperature (C°)	10.9	
	Conductivity (µS/cm)	345	
	Secchi disk (m)	6.7	
Chemical	Total N (mg N/L)	1.59	
	NO_3 (mg N /L)	1.52	
	NH_4 (mg N /l)	0.03	
	TP (mg P/l)	0.06	
	TOC (mg C/l)	1.61	
	Chl-a surface (µg/L)	1.82	
Metabolism		**Net Production (g $O_2/m^2/h$)**	**Respiration (g $O_2/m^2/h$)**
	Sparganium emersun	1,296	-15,769
	Groenlandia densa	18,654	-267,939
	Chara hispida	4,199	-4,324
	Chara vulgaris	3,396	-9,362
	Sediments	na	-21

$\delta^{15}N$ tracer addition

Baselines values of $\delta^{15}NH_4$ showed $\delta^{15}N$ signatures between 20 and 30 ‰. After tracer addition the ^{15}N label to the lake greatly enriched the $^{15}NH_4$ pool in the lake and downstream pools with maximum peaks ~2,700 ‰ at 36 h and 60 h, respectively (Fig. 4.4a, Table 4.2). There were not detected any relevant

isotopic increase upstream during all the sampled days. $\delta^{15}NO_3$ natural abundances varied from -2.10 to -4.7 ‰ and after the ^{15}N addition results showed a modest $\delta^{15}NO_3$ increase in both the lake and downstream sites with maximum peaks of 60 and 14.6 ‰ at time 36 h (Table 4.2).

Three weeks after the tracer addition, $\delta^{15}NH_4$ and $\delta^{15}NO_3$ values almost recovered baseline magnitudes (Fig. 4.4). All samples during the experiment showed extremely low $\delta^{15}N_2$ values, close to 0 (Table 4.2). The amount of N_2 ranged from 0.86 to 2.8 μmoles, in the order of magnitude of what we expected (~0.9 μmoles; Fig. 4.5a). Baseline N_2O samples showed similar $\delta^{15}N$ values in the lake (-2.46 ‰) and downstream samples (-6.04‰), but higher $\delta^{15}N$ signatures were obtained upstream (20.38‰, Table 4.2).

Before and after the ^{15}N addition, samples showed similar patterns, with low $\delta^{15}N_2O$ values and N_2O mass amounts in concondance with expected values (~400 pmoles) in the lake and downstream samples, and higher $\delta^{15}N_2O$ signatures and lower N_2O mass in upstream samples (Table 4.2, Fig. 4.5b). Overall, a negative relationship bewteen the N_2O mass and $\delta^{15}N_2O$ signatures was found (R^2= 0.73).

Baseline $\delta^{15}N$ values found in the sediments ranged from 3.5 to 7.1 ‰. During all the experimental addition period, sediments showed similar $\delta^{15}N$ values, reflecting negligible ^{15}N enrichment (Fig 4.6). All the collected plants showed similar basal $\delta^{15}N$ values, varying from -2.6 to 5.4 ‰ (Table 4.2).

Table 4.2: $\delta^{15}N$ and $\delta^{13}C$ natural abundance stable isotope values (baseline) and maximum $\delta^{15}N$ (highest observed enrichment) over the addition experiment. Natural abundance values reported the standard deviations. The dash (na) indicates samples were not analyzed and asterisk (*) means there is only one sample and SD is cero.

Pool	Species/compound	Habitat	Baseline $\delta^{15}N$ (‰)	SD	Maximum $\delta^{15}N$ (‰)
Dissolved Inorganic Nitrogen	NH_4	Upstream	21.55	2.93	209.09
		Lake	31.27	*	2776.11
		Downstream	25.10	*	2492.95
	NO_3	Upstream	-2.10	*	-2.10
		Lake	-3.00	*	61.00
		Downstream	-4.77	*	14.60
	N_2	Upstream	-1.58	0.83	0.22
		Lake	-0.91	0.03	0.65
		Downstream	-0.35	0.82	-0.01
	N_2O	Upstream	20.38	10.84	40.67
		Lake	-5.44	2.68	-1.57
		Downstream	-6.04	1.84	-0.67
Primary Producers	*Groenlandia densa*	G1	0.38	0.46	67.09
		G2	-2.21	0.45	60.47
	Chara vulgaris	V1	-1.66	0.34	36.15
		V2	-1.18	1.38	34.73
		V3	na	na	54.75
	Chara hispida	H1	-0.74	0.15	30.74
		H2	1.49	0.66	34.82
	Sparganium emersum	H1	-2.59	0.57	14.62
		H2	4.38	0.20	18.64
	Cladium mariscus	All sites	6.60	2.05	6.80
	Phragmites australis	All sites	6.10	0.27	6.40
	Juncus inflexus	All sites	4.20	1.62	5.00
Sediments		Upstream	3.72	0.13	4.95
		SED1	5.90	2.02	3.27
		SED2	3.53	0.16	4.62
		SED3	7.14	0.24	5.36
		SED4	3.91	0.18	5.04
		Downstream	4.31	0.10	6.76

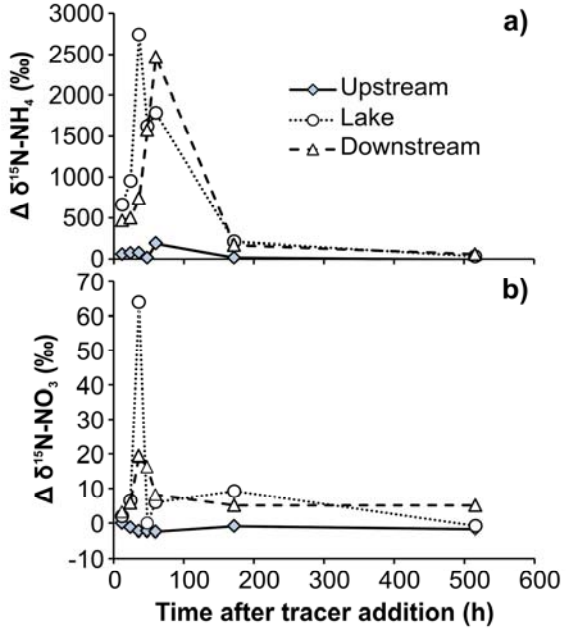

Fig. 4.4: Temporal profiles of observed $\Delta^{15}N$ labels of (a) NH_4 and (b) NO_3 pools in the 3 study sites of Somolinos. Values are expressed as increments from the baseline values (Δ). Time are expressed in hours.

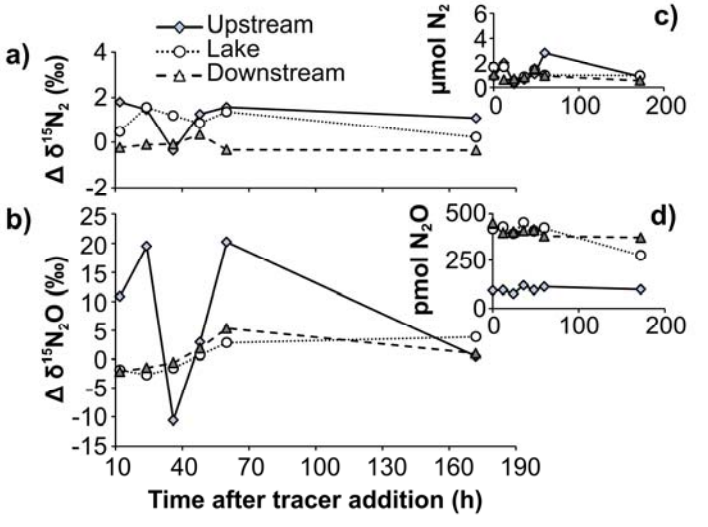

Fig. 4.5: Temporal profiles of observed $\Delta^{15}N$ labels of a) N_2 and b) N_2O pools in the 3 study sites of Somolinos.c) N_2 and d) N_2O mass values (expressed as µmoles and picomoles) *vs*. the time are graphed in the right corner.

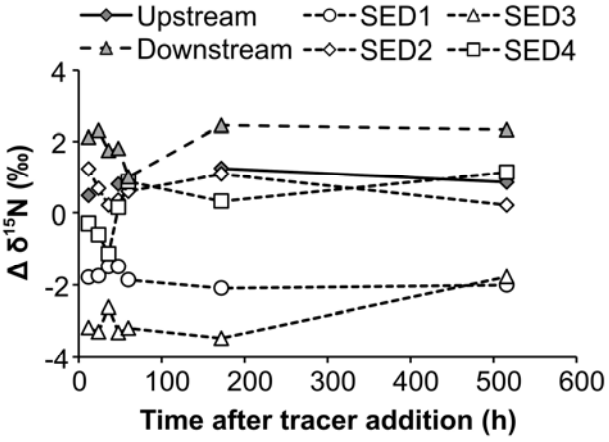

Fig. 4.6: Temporal profiles of observed $\Delta^{15}N$ labels and representative spatial distribution of the sediment samples in Somolinos.

Results suggested overall $\delta^{15}N$ enrichment in all macrophytes species (Fig. 4.7). The highest and quick enrichment was observed in *Groenlandia densa* in all locations, showing a peak of 74 ‰ within the first 40 h after the isotope addition (Fig. 4.7a). In this species, $\Delta\delta^{15}N$ decreased with the time, but still remained high after three weeks of the ^{15}N tracer addition. $\Delta\delta^{15}N$ observed in *Chara vulgaris* tended to progressively increase up to 30 ‰ during the first week, followed by a moderate decrease which did not decrease significantly the enrichment after the third week (Fig. 4.7b). Specimens collected in the left site (V2) showed a faster $\Delta\delta^{15}N$ enrichment but it decreased, reaching minimum values within the first week. *Chara hispida* showed similar $\Delta\delta^{15}N$ patterns, with a maximum of 30 ‰ after one week and remaining enriched values at the end of the experiment (Fig 4.7c). *Sparganium emersum* was the lowest ^{15}N labeled plant species, with $\Delta\delta^{15}N$ below 20 ‰ (Fig. 4.7d). Specimens of *S. emersum* collected in the middle of the lake (S2) remained enriched after the third week, whereas samples collected from the left littoral seemed to decrease faster, recovering basal $\delta^{15}N$ values for then. Helophytes

(*Cladium mariscus*, *Phragmites australis* and *Juncus inflexus*) $\delta^{15}N$ values before and at the third week were typical from natural ^{15}N abundances (4-9 ‰), so we did not observe any $^{15}\delta$ increment (Table 4.2).

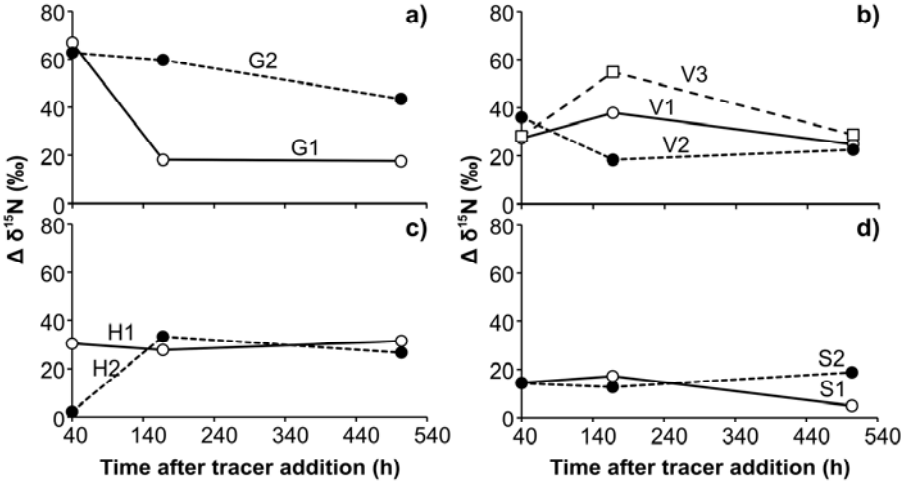

Fig. 4.7: Temporal profiles of observed $\Delta^{15}N$ labels and representative spatial distribution of primary producers: a) *Groenlandia densa, b) Chara vulgaris,* c) *Chara hispida and d)Sparganium emersun*. Letters G, V, H and S mean the different locations of each collected samples (see map in Fig 4.1).

Calculations of ^{15}N fluxes of $^{15}NH_4$, $^{15}NO_3$ and macrophytes species within the first 172 h are shown in Table 4.3. Nitrification rate in the water column was calculated at 36 h, coinciding with the maximum $\delta^{15}NO_3$ and was estimated to be 0.5 nmol N/l day. From the total ^{15}N added to the system (157 g), 1.12 % still remained as NH_4 after 172 h of starting tracer addition, 5.3 % was transformed to NO_3 in the lake water and 14 % was storage in the macrophyte biomass (Table 4.3).

Table 4.3: Mass balance of ^{15}N in the sampled pools. Calculations were done with collected data from the time 172 h.

Pool	g ^{15}N	% ^{15}N
NH_4	1.76	1.12
NO_3	8.38	5.34
Sparganium emersum	0.21	0.13
Chara hispida	19.24	12.25
Groenlandia densa	1.45	0.93
Chara vulgaris	0.64	0.41
Total added	157.00	100.00
Total accounted for	31.69	20.18
Not account for	125.31	79.82

Macrophytes exhibited the higher N short-term fluxes over all the sampled compartments, retaining a total of 637 g N /h. Averaged macrophyte NH_4 uptake rates (mg N/ m^2 day) and turnover times (days) for each of the four macrophyte species are summarized in Table 4.4. *Chara hispida* showed the highest retention rates and, although the exactly turn over value could not be calculated because the equilibration to baseline values was not obtained after 3 weeks of addition, it was expected to be the longest one.

Table 4.4: Biomass (g/m^2), total nitrogen content (g N/ m^2), uptake (g N/ m^2d), turnover rate (h^{-1}) and turnover time (d) for each of the submerged macrophytes species in Somolinos lake. Turnover of chara hispida was not calculated (nc), because at the end of the experiment, $\Delta\delta^{15}N$ remained very high values.

Pool	Biomass (g/m^2)	TN (g N /m^2)	NH_4 uptake rate (mg N /m^2 d)	Turnover rate (h^{-1})	Turnover time (d)
Groenlandia densa	366	9.43	296.53	0.002	20.8
Chara vulgaris	516	10.51	311.94	0.001	41.6
Chara hispida	1331	31.46	1019.1	nc	nc
Sparganium emersum	75	1.79	25.62	0.005	8.3

4.5 DISCUSSION

$\delta^{15}NH_4$ natural abundances were in concordance of signatures found in other studies as Cifuentes et al. (1989), Velinsky and Fogel (1999) and Middelburg and Nieuwenhuize (2001). $\delta^{15}NO_3$ natural abundances also showed values in agreement with the ranged found in the literature (~ -6.4 to 10 ‰; Middelburg and Nieuwenhuize, 2001; Piatek et al., 2005). Low $\delta^{15}N_2$ signatures observed in the three sampling stations during all the enrichment experiment give us evidence of an inadvertent contamination by atmospheric N_2 introduced during sampling as reported in other ^{15}N enrichments studies (e.g. Tobias et al., 2001). Regarding the N_2O pool the low $\delta^{15}N_2O$ signatures in all the samples and the negative relationship bewteen the N_2O mass and $\delta^{15}N_2O$ signatures suggested troubles either during the head-space sampling method or during sampling analyses by poor analytical precision. Under this circumstances, N_2 and N_2O pools were not accounted in our study to avoid any misleading conclusion. Baseline $\delta^{15}N$ values associated to sediments and macrophytes compartments were alike the magnitude found by other studies in lake sediments (Terranes and Bernasconi, 2000; Torres et al., 2012) and similar macrophytes species (Cifuentes et al., 1988; Fry, 1991; Cole et al., 2004; Inglett and Reddy, 2006).

N mass balance and N fluxes

Our ^{15}N experiment gives evidences that in oligotrophic lakes such as Somolinos lake, ammonium is either, rapidly converted to NO_3, assimilated by macrophytes or transfer throughout the ecosystem, being released downstream. One week after the whole-lake ^{15}N enrichment, the NO_3 pool showed 4 times more ^{15}N than the NH_4 pool, giving evidence of the importance of nitrification in this oligotrophic lake. Considering the entire system, the averaged

nitrification rate (0.5 nmol N/l day) is lower according to values reported in other lakes (Small et al., 2014), but in the range of rates found in pelagic environments from oligotrophic zones of the Pacific and Atlantic oceans (e.g. Raimbault et al., 1999; Clark et al., 2008).

Because the system exhibited a short residence time (2 weeks), a large fraction of the ammonium added into the system was lost through the outflow. A small fraction must be retained through sedimentation according with the low sedimentation rates registered, compared with other similar lakes (0.009-0.09 g N/m^2 day in La Colgada Lake; Piña et al., 2006). Our results showed, however, that macrophytes exhibit a moderate rapid uptake of ammonium (in a magnitude of days) compared to the values reported by Epstein et al. (2012), which showed slow (in a magnitude of weeks) and moderate ^{15}N labeled associated to macrophyte species after a ^{15}N injection in the Bull Trout Lake. Similar rates of ammonium incorporation that observed in our study have been reported in macrophyte incubations and microcosm experiments (e.g., Vermeer et al., 2003; Rodrigo et al., 2007). Nitrogen uptake rates for *Groenlandia densa* and *Chara vulgaris* showed similar values (1.21-3.86 μM/g DW h) than those reported for *Chara hispida* in Colgada Lake (Rodrigo et al., 2007). However, in Somolinos Lake, *Chara hispida* reported greater values, in the order of 95 μM/g DW h. This discrepancy among the values observed in *Chara hispida* can be related with greater biomass, but also because in Rodrigo et al. (2007) N uptake rate was related to nitrate and not ammonium, and our data suggested a preference of NH_4 over the NO_3 source in this lake, as observed in other studies (Vermeer et al., 2003). Moreover, our results also agree with those authors that emphasize the ability of submerged macrophyte beds to act as nutrient sinks in lakes (Kufel and Kufel, 2002; Rodrigo et al., 2007) and streams (Peipoch et al., 2013). Slow decomposition rate, which we implied by

the observed long turn over times (> 40 days in charophytes), means that N trapped in the macrophyte tissues will be retained for a relatively long period. Particularly, charophytes have been reported to decompose slower than other vascular aquatic plants growing in lakes, prolonging nutrient storage in their biomass (Kufel and Kufel, 2002).

N uptake by macrophytes

When the lake is viewed as an integrated whole system, the possibility for primary producers arises from the use of the resources of the pelagic *versus* the benthic habitat (Vandeboncoeur et al., 2002). Submerged macrophytes, such Charophytes and *Sparganium emersum,* have been reported to take up nitrogen from both water and sediment (Box, 1986, 1987; Barko et al., 1991; Vermeer et al., 2003; Meire et al., 2006) through the shoots and the roots or rhizoids. Since sediments have been generally thought to be the main source of N, most of these studies concluded that macrophytes would primary obtain the N from this pool, rather than direct assimilation from the water column (Barko et al., 1991; Granéli and Solander, 1988; Wetzel et al., 2001). However, our study evidenced a quickly incorporation of N into the macrophytes through water from DIN, suggesting a preferentially short-term N uptake via pelagic habitat instead of the benthic pool. These results are in concordance with Best and Mantai (1978) and Touchette and Burkholder (2000), who concluded that macrophytes may absorb more rapidly the NH_4 present in the water than in the sediments, resulting in the accumulation of N in the leaves. The relative importance of pelagic *vs* benthic N source in the N uptake have been also related with the availability of nutrients in the water column *versus* the sediment, on the nutritional status of the cells with respect to N, on the growing

status of the plants and own plant species physiological strategies (Carignan 1982; Carr and Chambers 1998; Eugelink 1998; Vermeer et a., 2003; Lee et al. 2007). Our results on N uptake rates revealed these differences in species functionality: for example, *Groenlandia densa* showed a much higher N uptake rate (per unit) than *Sparganium emersum*, even though both showed similar N content in their tissues. This attribute make *Groenlandia densa* more efficient in assimilating N directly from the water, while *Sparganium emersun* would preferentially obtain the N from the sediment. Despite of these clear evidences of N uptake directly from the pelagic zone, it should be remarked that our results also suggested the possibility of N uptake through sediments, probably in a long-term scale. $\delta^{15}N$ natural abundances found in the macrophytes species were in a similar range of those in the sediments, which may related both pools as N source-consumer (Peterson and Fry, 1987). Indeed, the values are closer in the case of *Sparganium emersum* and in all the helophyte species (*Cladium mariscus, Phragmites australis* and *Juncus inflexus*), which are only partially cover by water, and presumably would preferentially assimilate the N from the sediment. Somehow similar results were reported recently by Peipoch et al. (2013), where they concluded that submersed macrophytes species contribute to in-stream N uptake by assimilation of DIN from the water column, while macrophyte species located at stream channel edges do seems to rely on hyporheic and/or groundwater rather than on stream water DIN. These authors related these differences mainly on the degree of water exposure and the structural traits of the different functional types of macrophytes. Our results could be suggesting that in oligotrophic lakes, where nitrogen is limiting, macrophytes possessing physiological ability to assimilated N from both water and sedimentary sources would be more successful in the competition for limited resources but under changes in the nutrient availability as occurring

during eutrophication this functional trait would make the plants more sensitive and vulnerable.

In sum, in oligotrophic lakes with low water retention times, as in Somolinos Lake, nitrogen processes are occurring quickly according to the water flow: N available must be rapidly transformed (microbially) or assimilated (macrophytes) before is transferred downstream. Since nitrate appears under low concentration in this lake type, ammonium availability probably is also controlling denitrification. Macrophytes act as temporal N sink, greatly defining the functioning of these lakes subjected to such rapid nutrient dynamics.

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CHAPTER 5

Abundance and distribution of functional genes encoding key enzymes in the N cycle of freshwaters

"The genes of organisms, to the point that influence in their behavior, physiology and morphology, are at the same time, helping to build their environment"

Richard Lewontin

5.1 SUMMARY

Along the land-to-ocean aquatic continuum, processes of nitrogen storage, transformation and removal are mainly controlled by microbial communities. In lakes and streams, tightly coupled nitrification-denitrification-annamox processes play key roles as major biotic mechanisms of loss for fixed nitrogen (N), whereas dissimilatory nitrate reduction (DNRA) is considered as positive feedback to conserve N in a biologically available form. Given the relevance of these biogeochemical transformations in freshwaters, in this study we explored these processes in the stream Arroyo Grande del Molinillo and the lake Somolinos by a functional-gene-based approach. To this end, we measured, by q-PCR, potential occurrences and abundances of functional genes that encode key enzymes of nitrification (*amoA*), denitrification (*narG, napA ,nirS and nirK, nosZ*), DNRA (*nfrA*) and Anammox (*16S* annamox) processes, in benthic and pelagic samples. Results from the genes abundance assays suggested that, overall, genes encoding the denitrification process dominated the total gene pool in both studied freshwater systems. Results also give evidence to suggest that biological demand via nitrification may be partly saturated in Arroyo Grande del Molinillo while, on the contrary, the coupled nitrification-denitrification processes would be of crucial importance in the removal of N from the Somolinos Lake. In both studied ecosystems, abundances of genes encoding enzymatic pathways of DNRA and Annamox appeared to be marginal.

Key words: functional genes, nitrogen cycle, q-PCR, freswhwater ecosystems

5.2 INTRODUCTION

Nitrogen (N) is constantly transformed and recycled in both microbially mediated processes, within and between aquatic ecosystems and the atmosphere. As it has been already discussed (chapters 3 and 4), microbial transformations of N compounds, through oxidative (i.e. nitrification, annamox) and reductive (i.e. denitrification, DNRA, ammonification and nitrogen fixation) are key processes in the global N flux (Herber et al., 1999). However, the ecological significance of some of these processes (e.g. anammox and denitrification in water column; Haersley, 2009; Hamersley, 2009; Yosinaga et al., 2011) and the abundance and distribution of some the responsible organisms (e.g. denitrifying organisms, Nogales et al., 2002) requires further study.

In aquatic ecosystems, denitrification was long thought to be the main metabolic pathway of N release to the atmosphere (Zumft, 1997; Scott et al., 2008), but today losses by anammox process are also recognized as an important budgets (Ward et al., 1996; Trimmer et al., 2003; Risgaard-Petersen et al., 2004; Seitzinger et al., 2006). In denitrification, a widespread variety of facultative anaerobic bacteria use nitrogen oxides as an alternative terminal electron acceptor during the oxidation of organic matter (heterotrophic denitrification) or inorganic matter (autotrophic denitrification) (Zumft 1997, Burgin and Hamilton, 2007). Denitrification can thus occur in any system where nitrate and organic matter are available (Seitzinger et al., 2006). In contrast to denitrifying bacteria, anammox is carried out only by autotrophic anaerobic bacteria that oxidate $NH_4^+$and/or NO_2^- to produce N_2 (Hu et al., 2011). Although bacteria conducting annamox have been detected in many natural ecosystems, their role has been investigated for only few environments,

mainly, marine ecosystems, estuaries and wetlands (Rysgaard et al., 2004; Arrigo, 2005; Kuypers et al., 2005; Meyer et al., 2005; Penton et al., 2006). Only recently the structure and composition of denitrifier and anammox bacterial communities in lake and river sediments and lake water column received more attention (Schubert et al., 2006; Perryman et al., 2008; Hamersley et al., 2009; Dale et al., 2009; Kim et al., 2011; Yoshinaga et al., 2011).

Nitrification is achieved through a two-step process involving two different chemolithotrophic bacterial groups: ammonia-oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) to produce nitrate (Risgaard-petersen et al., 2003). Dissimilatory nitrate reduction to ammonium (DNRA) is one of the least understood N processes, and most DNRA reporting studies so far have been conducted in coastal and estuarine ecosystems (e.g. Christensen et al., 2000, Jäntti et al., 2011). DNRA may prevail when O_2 is in short supply (Jäntti and Hietanen 2012) and in highly reducing conditions, as in sediments with high organic matter content and low NO_3^- availability (Christensen et al., 2000). DRNA conserves N within the ecosystem and there is evidence that it supposes a major pathway of N that cannot be ignored (An and Garder, 2002). This process is widely spread among bacteria which may be facultative anaerobes or not.

To understand the biogeochemical N cycle it is essential to know the abundance and distribution of microbial communities within the environments (Smith and Osborn, 2009). However, species composition analysis provides interesting information on community composition under certain environmental conditions but metabolic functions cannot be associated with a specific taxonomic group (e.g. nitrate reduction is mediated by a diverse

polyphyletic group of bacteria, and rRNA-based approaches are of limited value for understanding the structure and diversity of nitrate-reducing communities, Smith et al., 2007). Molecular ecology improves our understanding of microbial ecology and biogeochemistry through the analysis of genes encoding metabolic processes for ecosystems: specific functional gene abundances can be determined and the enzymatic capacity of the microbial communities assessed (Wagner and Loy, 2002; Throbäck et al., 2004; Philippot and Hallin, 2005).

Several analytical techniques have been described for functional genes assessment, including Fluorescence in situ hybridization (FISH, De Beer and Schramm, 1999), immunofluorescence probing (Hastings et al., 1998), denaturing gradient gel electrophoresis (Kowalchuk and Stephen, 2001), and polymerase chain reaction based technique (PCR, Smith et al., 2007). Whereas most of these methods can only be used under high microbial abundances, PCR-based methods are capable of detecting DNA/RNA at low concentrations (Zhang and Fang, 2005). Data generated by quantitative PCR method (q-PCR) can be used to relate variation in gene abundances and/or levels of gene expression in comparison with variation in abiotic or biotic factors and/or biological activities and process rates (Smith et al., 2009). Even the occurrence of these functional genes does not necessarily indicate that the corresponding bacteria display the expected activities (Philippot and Hallin, 2005) providing, anyway, an indirect useful measure of intrinsic enzymatic capacity for the particular biogeochemical processes within the aquatic ecosystems (Smith and Osborn, 2009; Graham et al., 2010). Consequently, q-PCR has been widely used as a quantitative approach to study variation in gene abundances that encode key enzymes involved in the N cycle in lakes (Kim et al., 2011; Auguet et al., 2011; Vila-Costa et al., 2014), streams (Huang et al., 2011; Boulêtreau et

al., 2014) and wetlands (Nogales et al., 2002; Smith et al., 2007; Graham et al., 2010).

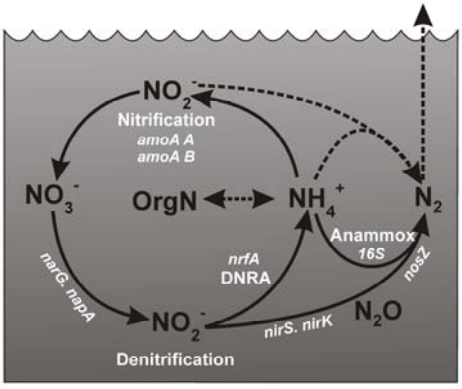

Fig. 5.1: Nitrogen transforming reactions and associated functional genes coding for enzymes utilized in different steps of the processes in an aquatic ecosystem.

The combination of isotope-based ^{15}N enrichments and DNA-based methods, such as molecular markers, have provided excellent results in the study of N dynamics in aquatic ecosystems (Kuypers, et al., 2003; kartal et al., 2007; Smith and Osborn, 2009). In this chapter, we explored this dual perspective by measuring the potential occurrence of N microbial activities in two freshwater ecosystems that were studied through ^{15}N *in situ* tracer experiments; Arroyo Grande del Molinillo stream (chapter 3) and Somolinos lake (chapter 4). To this end, we targeted the functional genes that encode key enzymes involved in the aquatic N cycle (Fig. 5.1): aerobic and anaerobic ammonia oxidation (*amoA* and *16S Annamox*), denitrification (narG, napA, nirS, nirK and nosZ) and dissimilatory nitrate reduction (*nfrA*). We assessed the gene abundance in sediments and water samples, determining the functional composition of microbial communities in each environment and

evaluating the relationships between microbial potential gene expression and nitrogen processes.

5.3 MATERIAL AND METHODS

Site descriptions and data collection

The study sites have been exhaustively characterized in chapter 3 (Arroyo Grande del Molinillo stream) and chapter 4 (Somolinos lake). Sediment samples -first 10 cm- were collected with a core sampler in 4 sites of the studied Arroyo Grande del Molinillo (25, 41, 115 and 175 m), and along a longitudinal transect in Somolinos Lake (inflow, S1, S2, S3, S4, outflow). All sediments were kept in 60 ml vials and preserved in ice. Since microbial communities are negligible in the water column of streams, water samples were collected only in Somolinos lake at 3 locations (inflow, middle lake and outflow), and in two time frames (before and after the ^{15}N enrichment). In each duplicate, the 1.5-2 liters of lake water sampled was filtered using membrane filters (0.22 μm). Triplicate filters and sediments samples were analyzed in the Laboratorio of the Molecular Microbial Ecology at Universitat de Girona, for q-PCR methodology.

DNA extraction and PCR amplification

Nucleic acids of each sample were extracted using *FastDNA Spin Kit for Soils (MP Biomedicals)* according to the manual of the manufacturer. Then, samples were quantified by *Qubit® 2.0 Fluorometer (High Sensitive kit)(Life Technologies)* and stored at -20ºC. Quantitative PCR (q-PCR) amplification

was run for the target genes: *amoA B* (associated to bacteria community), *amoA A* (associated to archaea community), *narG, napA ,nirS, nirK*, *nosZ*, *nfrA* and *16S rRNA annamox*, and the total bacterial and archaeal community was quantified using the *16S rRNA* gene. All used target genes and their associated primers have been already developed to be used as molecular markers of the different functional groups (Table 5.1). Reactions were carried out in a 7500 Real Time PCR system (Applied Biosystems) using the SYBR Green PCR Mastermix (Applied Biosystems) and following primers and thermal cycling conditions described in Table 5.1. Reactions were performed in a final volume of 20 µL of reaction mixture containing 2 µl of sample (10 ng of template genomic DNA), 1 µl of each primer (at 20 µM concentration), 2 µl of autoclaved water, 2 µl of $MgCl_2$ (at 25mM concentration), 2 µl of BSA (at 10 mg/ml concentration) and 10 µl of SYBR Green Master Mix (at 10X). Assays were run in triplicate including negative controls and standard curves spanning from 10^2 to 10^8 copies of the respective genes (r^2>0.99 for standard curves). Overall, average efficiencies were >80 %. Expected size of qPCR products and absence of unspecific PCR products were verified by 1.5% agarose gel electrophoresis and visualized through ethidium bromide staining.

Statistical Analyses

Gene abundances were analyzed as absolute abundance values. Normality of the data was tested with Kolmogorov-Smirnov goodness-of-fit test (STATISTICA v.6). Values from q-PCR results were log-transformed to fit normal distribution. Differences between variables were assessed by one-way ANOVA and a post hoc test for mean comparison (Tukey HSD) using STATISTICA.

5.4 RESULTS

Spatial distribution and gene abundance in Arroyo Grande del Molinillo

Total bacterial community *(16S rRNA)*, denitrifier *(narG, napA ,nirS, nirK and nosZ*), nitrifier (*amoA B* and *amoA A*) and anaerobic ammonium oxidizer (*16S Annamox*) functional genes were detected in all the sediment samples (Table 5.2). Dissimilatory nitrate reduction functional group (*nfrA)* abundances were below the detection limit in all the samples. Total bacterial community abundances, based on *16S rRNA* genes, ranged from 5.20 x 10^8 to 1.05 x 10^9 copy/g dw, being significantly lower in the station 25 m (Turkey´s HSD, $p < 0.05$) (Fig. 5.2).

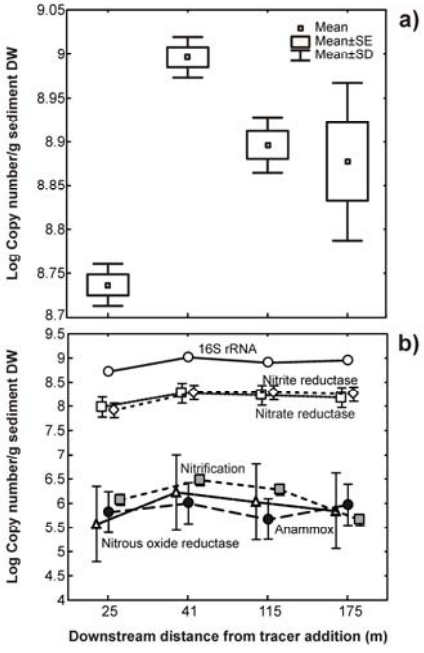

Fig. 5.2: a)Box and Wisker plot with the 16S rRNA genes abundances in Arroyo Grande del Molinillo. Y axis indicated the sampled stations: at 25, 41, 115 and 175 meters downstream,. b) mean values of abundances of functional genes in the sediments, vertical bars denote 0.95 confidence intervals.

Table 4. 1: p-PCR primers and probes targeting genes encoding enzymes involved in nitrogen cycling.

Functional group	Target gene	Primer	Sequence (5' – 3')	Thermocycle conditions	References
Total bacterial and archaeal community	*16S rRNA*	341F	CCT ACG GGA GGC AGC AG	95°C, 10 min, 1 cycle 95°C for 15 s, 60°C for 30 s, 72°C for 35 s, 40 cycles	Lopez-Gutiérrez et al. (2004)
		534R	ATT ACC GCG GCT GCT GGC A	95°C for 15 s, 60 to 95°C, 1 cycle	
Nitrite reduction	*nirK*	nirK876	ATY GGC GGV CAY GGC GA	95°C, 10 min, 1 cycle 95°C for 15 s, 63 to 58°C for 30 s (-1°C by cycle), 72°C for 30 s, 6 cycles	Henry et al. (2006)
		nirK1040	GCC TCG ATC AGR TTR TGG TT	95°C for 15 s, 60°C for 30 s, 72°C for 35 s, 40 cycles 95°C for 15 s, 60 to 95°C, 1 cycle	
Nitrite reduction	*nirS*	nirSCd3aFm	AAC GYS AAG GAR ACS GG	95°C, 15 min, 1 cycle 95°C for 15 s, 65 to 60°C for 30 s (-1°C by cycle), 72°C for 45 s, 6 cycles	Smith et al. (2007)
		nirSR3cdm	GAS TTC GGR TGS GTC TTS AYG AA	95°C for 15 s, 60°C for 30 s, 72°C for 1 min, 40 cycles 95°C for 15 s, 60 to 95°C, 1 cycle	
Nitrous oxide reduction	*nosZ*	nosZ2F	CGC RAC GGC AAS AAG GTS MSS GT	95°C, 15 min, 1 cycle 95°C for 15 s, 65 to 60°C for 30 s (-1°C by cycle), 72°C for 30 s, 80°C for 15 s, 6 cycles	Henry et al. (2006)
		nosZ2R´	CAK RTG CAK SGC RTG GCA GAA	95°C for 15 s, 60°C for 30 s, 72°C for 30 s, 80°C for 15 s, 40 cycles 95°C for 15 s, 60 to 95°C, 1 cycle	
Nitrate reduction	*narG*	narG-F	TTC GCC SAT YCC GGC SAT GTC	95°C, 10 min, 1 cycle 95°C for 30 s, 63 to 58°C for 30 s (-1°C by cycle), 72°C for 30 s, 6 cycles	Smith et al. (2007) Bru et al. (2007)
		narG-R	GAG TTG TAC CAG TCR GCS GAY TCS G	95°C for 30 s, 58°C for 30 s, 72°C for 35 s, 40 cycles 95°C for 15 s, 60 to 95°C, 1 cycle	

Table 4. 1: (continued)

Functional group	Target gene	Primer	Sequence (5' – 3')	Thermocycle conditions	
Nitrate reduction	*napA*	napA-V17m	TGG ACV ATG GGY TTY AAY C	95°C, 10 min, 1 cycle 95°C for 30 s, 61 to 56°C for 30 s (-1°C by cycle), 72°C for 30 s, 6 cycles	Smith et al. (2007) Bru et al. (2007)
		napA-4r	ACY TCR CGH GVG TRC CRC A	95°C for 15 s, 58°C for 30 s, 72°C for 35 s, 40 cycles 95°C for 15 s, 60 to 95°C, 1 cycle	
Annamox	*Amx*	A438f	GT CRG GAG TTA DGA AAT G	50ºC, 2min, 95°C, 10 min, 1 cycle 95°C for 30 s, 55,5°C for 15 s, 72°C for 35 s, 40 cycles	Humbert, Zopfi, and Tarnawski (2012)
		A694r	ACC AGA AGT TCC ACT CTC	95°C for 15 s, 60°C to 95°C, 1 cycle	
Nitrification	*amoA(AOB)*	amoA-1F	GGG GTT TCT ACT GGT GGT	95°C, 15 min, 1 cycle 94°C for 30 s, 55°C for 45 s, 72°C for 30 s, 35 cycles	Rotthauwe et al. (1997)
		amoA-2R	CCC CTC KGS AAA GCC TTC TTC	95°C for 15 s, 60°C for 30 s, to 95°C for 15 s, 1 cycle	
Nitrification	*amoA(AOA)*	CrenamoA23F	ATG GTC TGG CTW AGA CG	95°C, 15 min, 1 cycle 94°C for 30 s, 55°C for 45 s, 72°C for 30 s, 35 cycles	Tourna et al. (2008)
		CrenamoA616r	GCCATC CAT CTG TATGTCCA	95°C for 15 s, 60°C to 95°C, 1 cycle	

Table 5.2: Absolute gene abundance levels in DNA extracts from sediment and water surfaces of Semolina's lake. Values are means standard errors of the gene abundance levels (in copies/g [dry weight]) obtained from three independent DNA extracts from the sediments and water surfaces .(±) standard deviation; (*) undeterminated, below the standard values.

			Total community	Nitrate reductase		Nitrite reductase		Nitrous oxide reductase	DNRA	Annamox	Nitrification		
Somolinos	**Sediments (copies/g)**	**Station**	**16S rRNA (x 10^9)**	**narG (x 10^6)**	**napA (x 10^8)**	**nirK (x 10^7)**	**nirS (x 10^7)**	**nosZ (x 10^7)**	**nrfa (x 10^4)**	**16S (x 10^4)**	**amoA B (x 10^6)**	**amoA A (x 10^6)**	**R amoA B (%)**
		Upstream	3.5 ± 2.5	0.6 ± 0.1	1.2 ± 0.5	1.9 ± 0.6	3.9 ± 1.8	2.3 ± 0.4	4.0 ± 0.1	2.0 ± 0.8	0.6 ± 0.2	37.1 ± 27.4	2
		Downstream	3.4 ± 1.6	1.9 ± 1.5	2.9 ± 1.8	2.7 ± 0.2	18.0 ± 3.1	6.4 ± 0.8	4.9 ± 0.6	177.1 ± 93.7	3.7 ± 1.4	24.2 ± 10.4	14
		Lake-S1	0.7 ± 0.2	1.4 ± 0.8	0.8 ± 0.2	0.4 ± 0.1	1.8 ± 1.2	0.4 ± 0.0	2.7 ± 1.5	4.2 ± 2.9	0.4 ± 0.1	0.2 ± 0.0	67
		Lake-S2	0.7 ± 0.3	2.6 ± 2.2	0.8 ± 0.3	0.6 ± 0.2	1.6 ± 1.1	0.7 ± 0.4	2.7 ± 2.0	4.6 ± 2.6	0.2 ± 0.1	0.1 ± 0.1	73
		Lake-S3	0.9 ± 0.1	1.2 ± 1.1	1.6 ± 0.5	0.8 ± 0.1	3.4 ± 1.0	0.4 ± 0.0	2.5 ± 0.6	2.2 ± 1.0	0.3 ± 0.1	0.1 ± 0.0	83
		Lake-S4	0.9 ± 0.2	0.9 ± 1.1	1.6 ± 0.1	0.8 ± 0.2	3.3 ± 0.5	0.4 ± 0.1	2.7 ± 0.5	6.7 ± 4.3	0.3 ± 0.1	0.1 ± 0.0	82
	Water (copies/ml)		**16S rRNA (x 10^5)**	**narG (x 10^2)**	**napA (x 10^3)**	**nirK (x 10^2)**	**nirS (x 10^2)**	**nosZ (x 10^2)**	**nrfa (x 10^1)**	**16S (x 10^1)**	**amoA B (x 10^2)**	**amoA A (x 10^1)**	**R amoA B (%)**
		Upstream	0.1 ± 0.1	1.2 ± 0.6	1.0 ± 1.2	3.5 ± 3.3	2.7 ± 2.1	4.2 ± 3.0	0.9 ± 0.2	3.7 ± 1.5	1.0 ± 0.5	0.7 ± 0.5	95
		Downstream	2.8 ± 2.6	2.7 ± 0.4	0.9 ± 0.1	9.5 ± 1.5	1.6 ± 0.5	9.5 ± 0.4	1.8 ± 0.2	8.9 ± 0.3	2.7 ± 1.4	3.2 ± 2.2	94
		Lake	5.2 ± 2.9	1.8 ±0.2	1.1 ± 0.3	12.1 ± 0.0	2.2 ± 0.7	7.0 ± 2.5	2.3 ± 0.9	5.2 ± 3.6	3.7 ± 0.9	5.9 ± 4.2	96
Arroyo Grande del Molinillo	**Sediments (copies/g)**	**Station (m)**	**16S rRNA (x 10^8)**	**narG (x 10^6)**	**napA (x 10^8)**	**nirK (x 10^7)**	**nirS (x 10^8)**	**nosZ (x 10^6)**	**nrfa (x 10^1)**	**16S (x 10^5)**	**amoA B (x 10^5)**	**amoA A (x 10^5)**	**R amoA B (%)**
		25	5.4 ± 0.3	1.5 ± 0.3	1.1 ± 0.2	3.1 ± 0.0	0.7 ± 0.3	0.3 ± 0.3	*	6.5 ± 1.9	7.6 ± 2.2	8.1 ± 0.5	49
		41	9.9 ± 0.5	3.0 ± 0.2	2.1 ± 0.4	8.1 ± 0.1	1.3 ± 0.3	1.7 ± 0.9	*	8.7 ± 3.1	29.6 ± 6.6	10.6 ± 0.1	74
		115	7.8 ± 0.5	2.6 ± 0.6	1.8 ± 0.5	6.5 ± 0.2	1.1 ± 0.2	1.2 ± 0.8	*	4.2 ± 3.4	13.0 ± 2.2	7.6 ± 0.0	63
		175	7.6 ± 1.5	2.8 ± 1.1	1.2 ± 1.4	3.0 ± 0.2	1.0 ± 0.5	0.7 ± 0.4	*	8.0 ± 3.6	2.6 ± 0.6	2.1 ±0.8	56

Genes that encoded denitrification enzyme nitrate reductase *(NapA and narG),* nitrite (*NirK and nirS*) and nitrous (*nosZ*) oxide reductases showed values ranging 7.04 x 10^7 - 2.52 x 10^8 copy/g dw, 3.44 x 10^7 - 2.14 x 10^8 copy/g dw and 8.56 x 10^4 - 2.89 x 10^6 copy/g dw respectively, with similar overall abundances in all the sampling stations (Turkey´s HSD, $p < 0.05$) (Table 5.2). In agreement with our results, the nosZ abundance in soils is often lower than that of other denitrifying genes (Henry et al., 2006; Hallin et al., 2009). Relative abundances of the denitrifying genes were in good agreement with previous studies where the ratio of denitrification bacteria to total bacteria was reported to range from 0.1% to 5.0% (Tiedje, 1988; Cheneby et al., 2000; Chon et al., 2011). Annamox functional group (*16S rRNA Anammox*) abundances ranged from 1.91 x 10^5 to 1.20 x 10^6 copy/g dw. Nitrifiyer abundances ranged from 1.76 x 10^5 to 3.96 x 10^6 copy/g dw associated to the bacterial community and 1.15 x 10^5 to 7.99 x 10^5 related to the archaea community. Overall, nitrifiers were significantly lower in the 175 m sampling station (Turkey´s HSD, $p < 0.05$). Ammonia-oxidizing microbial assemblages were dominated by AOB (bacteria), with ratios AOB/total nitrifiers greater than 50 % in sampling stations 41, 115 and 175 m (Table 5.2).

Functional groups nitrate and nitrite reductase dominated the total sediment gene pool, being significantly higher than nitrous oxide reductase, anammox and nitrifiers in all the sampling stations (Turkey´s HSD, $p < 0.05$) (Fig. 5.3a-d). Nitrifiers showed higher abundances than anammox functional group in sampling location 4 and 115 m (Turkey´s HSD, $p < 0.05$) (Fig. 5.3a-d). There were not any statistical differences among spatial distribution of oxide reductase and annamox and nitrifiers gene abundances (Turkey´s HSD, $p < 0.05$).

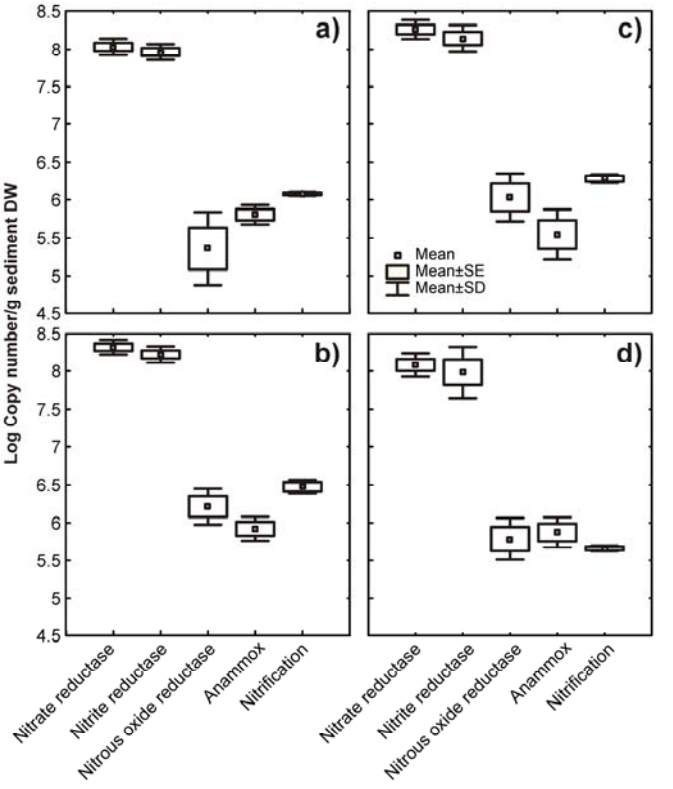

Fig. 5.3: Box and Wisker plot with the functional genes abundances in sediment samples of Arroyo Grande del Molinilo . Stream reaches: a) 25 m station, b) 41 m station, c) 115 m station and d) 175 m station. Abundances values are shown as log-transformed data.

Spatial distribution and genes abundance in Somolinos Lake

Sediment- Total bacterial and archaeal community (16S rRNA), denitrifier (*narG, napA ,nirS, nirK* and *nosZ*), dissimilatory nitrate reduction (*nfrA)*, nitrifier (*amoA A* and *amoA B*) and anaerobic ammonium oxidizer (*16S Annamox*) genes were detected in all sites (Table 5.2). Overall, abundances were highly variable, ranging several orders of magnitude (Table 5.2). In sediments, total community abundances (*16S rRNA)* ranged from 4.65 X 10^8 to

5.23 X 10^9 copy/g dw, being significantly higher in the inflow and outflow stations (upstream and downstream) and not statistically different within the lake´s samples (Turkey´s HSD, $p < 0.05$) (Fig. 5.4a).

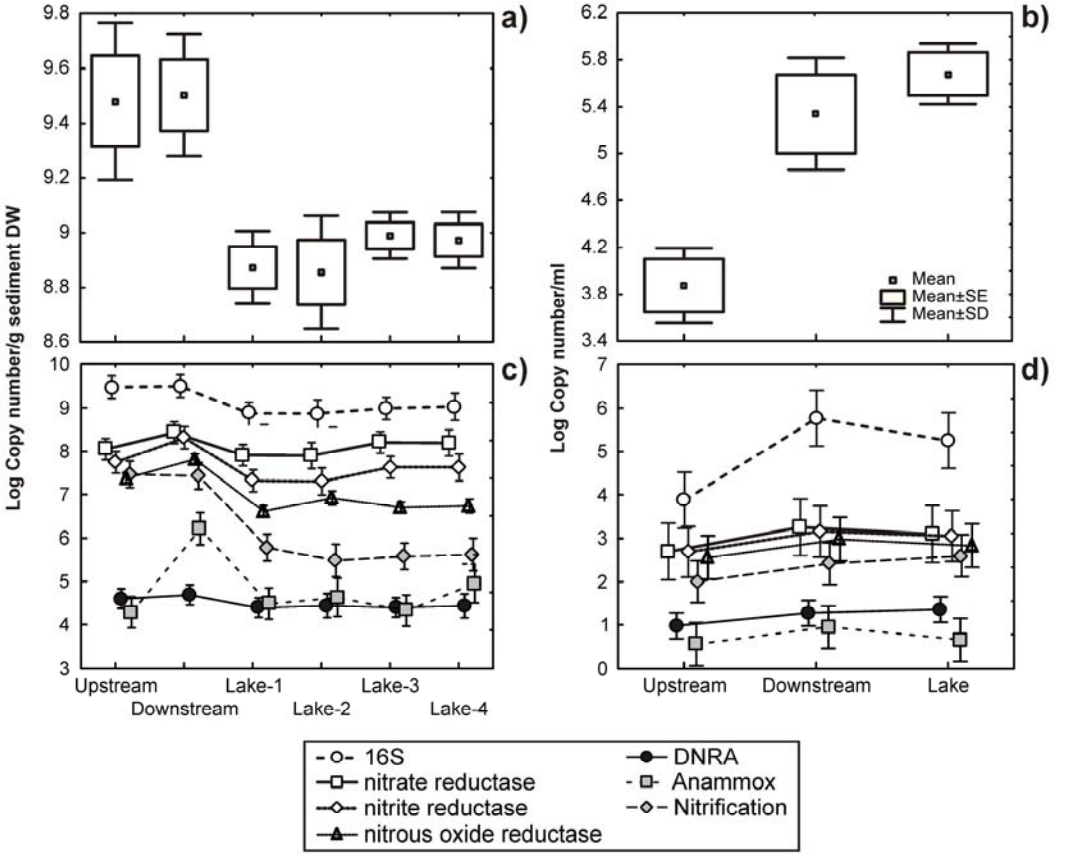

Fig. 5.4: Box and Wisker plot with the 16S genes abundances in Somolinos Lake. a) sediment samples, b) water samples. Overall abundances of functional genes in the sediments and water of Somolinos. Mean values are represented ,vertical bars denote 0,95 confidence intervals.

Genes encoding denitrification enzymes nitrate (*narG and NapA)*, nitrite (*nirS* and *Nirk*) and nitrous oxide reductase (*nosZ*) showed an average ranged from 4.97 x 10^7 to 5.04 x 10^8, 1.02 x 10^7 to 2.47 x 10^8 copy/g dw and 2.80 x

10^6 to 7.38 x 10^7 copy/g dw, respectively. Nitrite reductase abundances showed statistically higher values downstream compared to the other sampling stations, while nitrous oxide reductase showed higher abundances in downstream and upstream samples (Turkey´s HSD, $p < 0.05$) (Fig. 5.5). Abundances of functional group encoding DNRA (*nfrA)* ranged from 1.39 x 10^4 to 5.72 x 10^4 copy/g dw not showing statistical difference among sediment samples (Turkey´s HSD, $p < 0.05$) (Fig. 5.5). Annamox genes ranged from 8.33 x 10^3 to 2.85 x 10^6 copy/g dw, showing statistically higher values downstream (Turkey´s HSD, $p < 0.05$) (Fig. 5.4). Nitrifiers abundances ranged from 1.48 x 10^5 to 4.96 x 10^6 copy/g dw associated to the bacterial community and 2.84 x 10^4 to 6.38 x 10^7 copy/g dw related to the archaea community. Ammonia-oxidizing microbial assemblages were dominated by AOA (archaea) in stream samples (ratio AOB/total nitrifiers < 14 %) and by AOB (bacteria) in lake´s sediments (ratio AOB/total nitrifiers > 65 %) (Table 5.2). Overall, nitrifiers´ genes showed statistically higher abundances in downstream and upstream samples (Turkey´s HSD, $p < 0.05$) (Fig. 5.4).

Overall, genes that encoded the denitrification process dominated the total gene pool in sediments (Table 5.2). In lake sediments, nitrate and nitrite reductase were the most abundant, nitrous oxide reductase and nitrifiers showed intermediate abundances, whereas DNRA and annamox showed, statistically, the lower values (Turkey´s HSD, $p < 0.05$ Fig. 5.5c-f, Fig. 5.4). Downstream and upstream samples showed similar patterns of gene abundances, however, nitrifiers showed higher abundances (Fig. 5.5c-f, Fig. 5.4). In upstream samples, nitrifiers were not statistically different to denitrifiers and both functional groups dominated the gene pool (Turkey´s HSD, $p < 0.05$ Fig. 5.5c-f, Fig. 5.4).

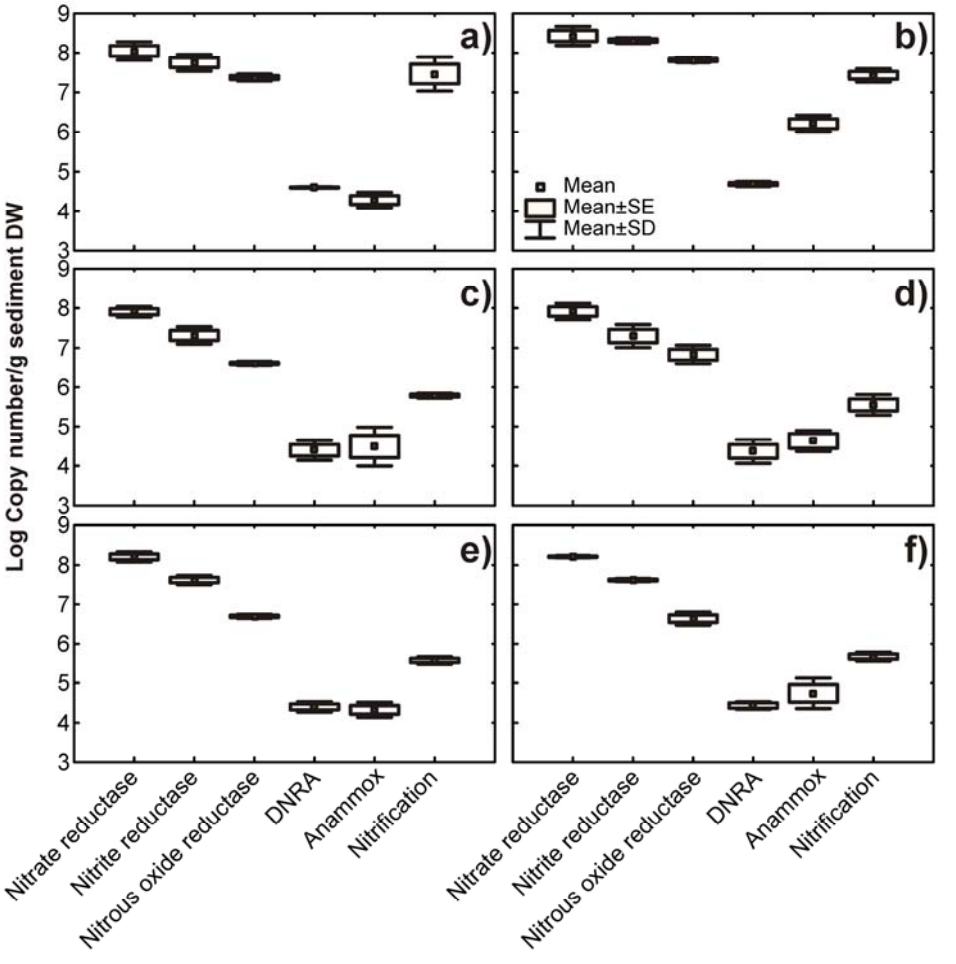

Fig. 5.5: Box and Wisker plot with the log-transformed functional genes abundances in sediment samples of Somolinos Lake. a) upstream, b) downstream c) Lake S1 d) Lake S2 e) Lake S3 f) Lake S4

Water column- In the water samples, total bacterial and archaeal community (*16S rRNA*), denitrifier (*narG, napA ,nirS, nirK* and *nosZ*), dissimilatory nitrate reduction (*nfrA)*, nitrifier (*amoA*) and anaerobic ammonium oxidizer (*16S rRNA annamox*) genes were detected in all sites (Table 5.2). Total community

abundances (*16S rRNA)* ranged from 4.43 X 10^3 copy/ml to 7.29 X 10^5 copy/ml, being significantly lower in upstream samples (Turkey´s HSD, $p < 0.05$, Fig. 5.4b). Nitrate (*narG* and *NapA),* nitrite (*nirS* and *Nirk*) and nitrous oxide reductase (*nosZ*) functional groups showed similar abundances, ranging from 2.22 x 10^2 to 2.04 x 10^3 copy/ml, 2.36 x 10^2 to 1.49 x 10^3 copy/ml and 2.06 x 10^3 to 9.86 x 10^2 copy/ml, respectively (Table 5.2, Fig. 5.6). Abundance of anammox (*16S Annamox*) ranged from 3.03 to 7.60 x 10^1 copy/ml and 2.66 to 9.13 copy/ml, respectively. *Amoa* values ranged from 6.50 x 10^1 to 4.46 x 10^2 copy /ml associated to the bacterial community and 7.15 x 10^1 to 5.92 x 10^1 related to the archaea community. The Ammonia-oxidizing microbial assemblages were dominated by amoA B (Bacteria) rather than by amoA A (archaea) in all the sampled sites, with a ratio amoA B/ total nitrifiers greater than 88% in all sites (Table 5.2). As the exception of *16S rRNA*, functional genes groups showed similar spatial pattern in the three sampling locations (Turkey´s HSD, $p < 0.05$).

Overall, denitrifiers (nitrate, nitrite and nitrous oxide reductase) showed significantly higher abundances than DNRA and annamox pools (Turkey´s HSD, $p < 0.05$, Fig. 5.6). Nitrifiers´ abundances showed intermediated values, being lower than nitrate reductase, but higher than DNRA and annamox functional groups (Turkey´s HSD, $p < 0.05$).

Comparing the functional genes abundances of sediment samples from the studied ecosystems (Arroyo Grande del Molinillo and lake Somolinos), results showed a higher abundance of archaea and bacterial community (16S rRNA), nitrous oxide reductase and nitrification genes pool in the inflow and outflow of Somolinos (Turkey´s HSD, $p < 0.05$). Lake Somolinos showed the lowest values of nitrite reductase and annamox gene abundances, whereas there were

no significant spatial distribution patterns in nitrate reductase abundances (Turkey´s HSD, $p < 0.05$).

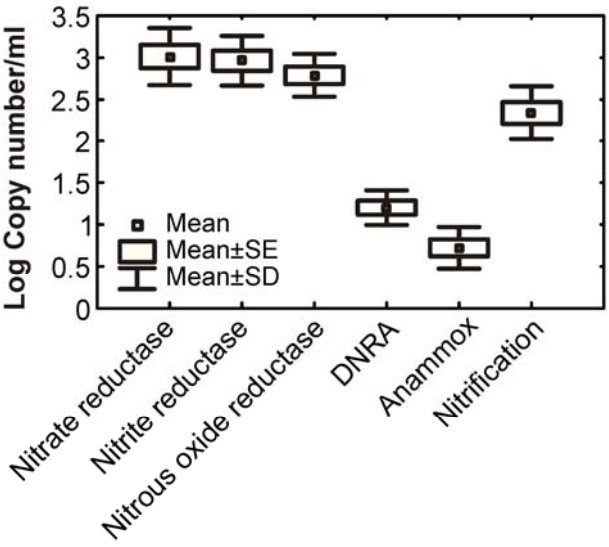

Figure 5.6: Box and Wisker plot with the functional genes abundances in water samples of Somolinos Lake. Samples from upstream , downstream and Lake collected sites were all compute in the same plot.

5.5 DISCUSSION

Spatial distribution of the functional genes might be influenced by nutrient conditions, type of sediment, accumulation of certain compounds, oxygen availability, and other environment variables (Huang et al., 2011).

Denitrification capacity in freshwater ecosystems

Since sediments accumulate organic and inorganic electron donors and acceptors, they have been considered the main spots of denitrification, thus high rates have been identified in streams and lake sediments (Vila-Costa et al., 2014). Even in oligotrophic lakes, such as Somolinos Lake, sediments may become anoxic within a few millimeters turning into suitable sites for denitrification (Vila-Costa et al., 2014). Abundances of denitrification genes in our studied sites were in the order of those found in other lake systems (Geets et al., 2007; Smith et al., 2007; Chon et al., 2011). These results indicated that denitrification occurs in sediments of Somolinos and Arroyo Grande del Molinillo and have considerable denitrification capacity. The occurrence of the assemblages of nitrite, nitrate and nitrous oxide reductase enzymes indicated that complete denitrification occurred in all the sediment cores. However, in Arroyo Grande del Molinillo, the low abundance of nosZ genes compared with the other denitrification genes is an indicative of the genetic capacity of the system to potentially accumulate the N_2O by an incomplete denitrification pathway.

The significance of denitrification in the water column remains partly unresolved because the few published instances of significant directly measured water column denitrification (Hamersley et al., 2009). Our results showed that denitrifying bacteria existed in the water column of Somolinos lake (with oxygen concentration values above 8.5 mg/L), indicating that, even though this is an apparently unsuitable condition, the notable gene richness suggests potential denitrification processes might occur in the water column. Indeed, denitrification is an oxygen-sensitive process, but it is carried out by

facultative bacteria that can also respire under oxic conditions (Vila-Costa et al., 2014).

In chapter 3 and 4 we discussed that we were not able to trace ^{15}N in the N_2 and N_2O pools as indicators of denitrification on both studied ecosystems. However, based on the q-PCR results, microbial denitrification may play an important role on the N cycle, being the main N removing pathway in these freshwater ecosystems (Boulêtreau et al., 2014). In Somolinos Lake (low-N system) denitrification contributes to N limitation by further decreasing N concentrations and by reducing the N:P ratio of recycled nutrients. According to Redfield et al. (1963), this N deficit resulting from denitrification gives rise to an excess of phosphate which tends to stimulate the growth of N_2 fixers. In last term, some studies has also proposed that in these N-limited systems, denitrification may have a critical role in the control of organic C sequestration by decoupling the N and C cycles (Falkowski, 1997). In Arroyo Grande del Molinillo, as it has been observed in similar streams with high dissolved inorganic N compounds levels (e.g., Mulholland et al., 2009; Graham et al., 2010), the removal of N by denitrification may reduce the export of N downstream, becoming a pivotal process that balances the flux of N into the biosphere and that can reduce the effects of N excess from the agriculture.

Ammonia oxidizing communities in freshwater ecosystems

The relative contributions of ammonia-oxidizing to the first step of nitrification (ammonia oxidation) have been investigated in aquatic ecosystems by determination of the abundance of archaeal- and bacterial-related *amoA* genes. Results from nitrifiyer abundances in the sediments of Arroyo Grande del

Molinillo, suggest that nitrification pathway seems to be partly saturated by chronic elevated NH_4 loading. Nitrifiyer abundances in the inflow, outflow and water column samples of Somolinos Lake suggest a stronger importance of the coupled nitrification-denitrification processes controlling N-cycling, as it has been already noticed in systems with low NO_3 concentration (Vila-Costa et al., 2014).

Some studies have suggested that archaea and not bacteria are the numerically dominant ammonia oxidizers in soil and seawater (Leininger et al., 2006; He et al., 2007; Mincer et al., 2007). Our results suggests an opposite trend with ammonia-oxidizing microbial assemblages in the sediments dominated by bacteria (AOB), as was also cited by Tada et al. (2011) and Correa-Galeote (2012) in sediments of constructed wetlands. Only in the stream inflow and outflow of Somolinos Lake, the microbial assemblages were dominated by archaea. This can be explained because spatial distribution of these communities may not be overlapped, showing different ecological niche variables, where their distribution may be related to environmental attributes such soil pH, clay content and ammonium levels (Limpiyakorn et al., 2011; Correa-Galeote, 2012). Our results encourage a re-evaluation of the basic understanding of nitrogen cycling and the relative importance of bacteria and archaea on nitrification processes.

Apart from the functional genes associated to denitrification and nitrification pathways, our q-PCR analyses also showed significant abundances of *16S rRNA Anammox* and *nrfa* genes, which encode enzymes related to Annamox and DNRA processes. Anammox has been shown to play a significant role in the elimination of N compounds from oceanic systems, but less attention has been paid to lacustrine ecosystems (Kartal et al., 2006;

Hamersel et al., 2009, Yosinaga et al., 2011). Gene abundances encoding annamox pathway in Arroyo Grande del Molinillo suggest a similar role than that of the nitrification process, whereas anammox bacteria were somewhat less abundant in sediments of Somolinos, and almost inexistent in the water column. In the water column, high rates of anammox have been reported, mainly, in strict anaerobic conditions (e.g. deeper zones of oceanic areas and stratified lakes, Jayakumar et al., 2013), which is not occurring at Somolinos Lake. Some authors suggest that *16S rRNA annamox* may not completely capture all the anammox diversity (Harhangi et al., 2013), and other functional genes have now been developed to complement this analysis (e.g., hydrazine synthase *hzsA*, Harhangi et al., 2013). Although a future evaluation using these complementary genes could provide new insights on this process, the absence of anoxic conditions in our studied lake suggest that anammox should be marginal in oligotrophic lakes.

The lack of data describing when and where DNRA occurs has been already highlighted (Burgin and Hamilton, 2007). Our results indicated that enzymes encoding the DRNA process was negligible in the Arroyo Grande del Molinillo and scarce in Somolinos Lake. Some authors suggested that DNRA occurs under more reducing (anoxic) conditions than the denitrification process (Page et al., 2003). However, there are not enough data available in the literature to make a comprehensive analysis and more studies are needed to systematically investigate the main controlling factors of DNRA in relation to environmental gradients.

In conclusion, our molecular assays suggested that, overall, genes that encoded the denitrification process dominated the total gene pool in both studied freshwater systems (stream and lake). N removal from the ecosystem

via denitrification and NH_4 uptake by macrophytes may be the main processes defining the whole N-cycle in these freshwater ecosystems. Whereas nitrification may be partly saturated by chronic NO_3 loadings as in Arroyo Grande del Molinillo, coupled nitrification-denitrification should remove N from the Somolinos Lake. Contrarily, in both studied ecosystems, DNRA and Annamox gene encodes appeared as marginal, indicating that the potential of both pathways may be negligible in the N cycle. While studies as this have greatly enhanced our understanding of microbial genes/processes in the environment, the next step requires an improvement of the link between variation in gene abundances with actual nitrogen processing rates in order to understand the N-cycling in aquatic systems and its importance on the global N-cycle.

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D. GENERAL DISCUSSION

This dissertation aimed to study the structure and nitrogen dynamics of inland waters by using different approaches derived from the use of the ^{13}C and ^{15}N stable isotopes. The first section of this general discussion is focused on the potential contributions of natural abundances and whole-ecosystem additions of these isotopes to elucidate the thesis objectives. The second section discusses the results derived from the use of ^{13}C and ^{15}N in studying the main environmental attributes and biotic assemblages as main factors defining the structure of lake ecosystems. Finally, the third section integrated the results from *in situ* deliberate ^{15}N enrichments and the measurements of enzymatic capacities regarding N dynamics in agricultural streams and oligotrophic lakes.

D.1 ASSESSMENT OF THE USE OF ^{15}N -^{13}C STABLE ISOTOPE APPROACHES IN THE STUDY OF FRESHWATER ECOLOGY: THE MORE, THE BETTER

The perspective of ecosystem-scale approaches in studying aquatic ecosystems considers a framework for the study of all biotic and abiotic components and processes and its interactions within defined boundaries (see Likens, 1992). The temporal stability of stable isotopes and the natural variations in $\delta^{15}N$ and $\delta^{13}C$ ratios as results from physical, chemical and biological processes (Craig, 1953, Fry, 2006), enables their potential use as biogeochemical tracers, allowing measurement of simultaneous ecological and biogeochemical processes at whole-ecosystem scale (Schimel, 1993; Fry, 2006).

In the last decades, many studies have highlighted the usefulness of $\delta^{13}C$ and $\delta^{15}N$ natural abundances to elucidate energy and matter flows in food webs and to integrate dietary pathways in aquatic ecosystems (e.g., DeNiro and

Epstein, 1978; Fry, 1991; Bearhop et al., 2004; Mildellburg, 2014). One of the advantages derived from this approach is that δ natural variations are generated, not only during primary production, but also in the subsequent heterotrophic processing, allowing the possibility to link small organisms at the base of the food web with large consumers at the top (Middelburg, 2014). While some studies typically display these $\delta^{13}C$ and $\delta^{15}N$ variations as x-axis and y-axis bi plots, in recent years, the sophistication of ecological requests called for a shift from qualitative treatment of SI data to more quantitative approaches (Schmid et al., 2007). In the first chapter of this thesis, we aimed to go further in the use of natural $\delta^{13}C$ and $\delta^{15}N$, by incorporating these isotopic data into Layman community-wide metrics to provide insights of lake´s structure. Although this approach has been already performed in previous studies to unravel temporal patterns on populations (e.g., Vanden Zander et al., 2002) or environmental attributes controlling food webs in similar lakes (e.g., Cooper and Wissel, 2012), the perspective of applying ^{13}C-^{15}N based Layman metrics as a tool to elucidate more global food web patterns within an ecosystem scale, rather than a single population, has been rarely carried out before. From this study we concluded that the application of Layman metrics provided more insights over the hypothesis regarding the structure of lake communities, for example, that lakes of greater diversity may contain tightly coupled functional groups, rather than a great linear food chain. However, our results also evidenced that Layman scores, by themselves, give poor information when they are applied at an ecosystem scale to derivate more general structural patterns (already discussed in Hoeinghaus and Zeug, 2008 and Syväranta et al., 2013). Therefore, today, interpretation of quantitative data obtained from SI approaches describing ecosystem structures require the complementary use other measurements, such as link and chain property based

food web descriptors. Although the application of this multi-approach perspective is often sufficient to resolve the SIA limitations in the study of food web patterns, the examination of other complex processes such as allochthonous/autochthonous consumer support, frequently required an alternative approach involving deliberate addition of SI at tracer level.

In the last two decades, SI tracer addition experiments at ecosystem scale have been widely used in aquatic environments, being incorporated as routine analyses in many laboratories (e.g. Tank et al., 2000; Mulholland et al., 2008; Middelburg et al., 2014). In the second chapter we provided a complete evaluation of the main *in situ* whole-ecosystem deliberate ^{13}C and ^{15}N enrichments that have been performed in freshwater ecosystems, paying special attention to their most relevant implications in the field of ecology and also their methodological limitations. From this full review, we concluded that ^{15}N and ^{13}C tracer additions at whole-ecosystem scale can be considered a powerful complementary tool to study some of the main ecological and biogeochemical processes within aquatic ecosystems. Moreover, our results from the field $^{15}NH_4$ additions performed in the stream Arroyo Grande del Molinillo and Somolinos Lake (Chapters three and four) gave great evidence that ^{15}N additions to aquatic ecosystems represent valuable advances in their study, shedding light on the internal functioning of the N cycle (e.g., agricultural streams may have long NH_4 uptake lengths and slow uptake velocities, partly because the system has surpassed the NH_4 threshold limits for natural recovery). Moreover, some of these ecological inquiries are often very difficult to assess without the application of SIA. However, despite these prolific advances in the field of ecology, we also concluded that we should be concerned and critical about some methodological and theoretical constraints hindering the wider use of this approach by aquatic scientists. We suggested

that some of these limitations can be easily solved by trial and error, such as the most appropriate compound to be used according to the experiment goal, the target level or the timing of injection and sampling; however, others, like the isotopic equilibrium, the lag between sources and foods and the isotopic fractionation, depend on a much broader framework and need a further revision of the isotope ecological theory.

The review of the literature concerning SIA and the observed results from the experimental research performed in this thesis gave support to the conclusion that both SI approaches have their advantages and drawbacks, and no one is preferable over the other. The main question resides in finding out the approach that fits better for the resolution of our specific ecological inquiries. Furthermore, numerous concerns and paradigms on aquatic ecology and biogeochemistry at ecosystem-scale are not sufficiently well known, such as metabolic rates and dynamics of microbial processes involved in aquatic N and C cycles, and we propose that the use of ^{13}C and ^{15}N approaches may be of help when addressing these present and future challenges, promising remarkable progress in aquatic sciences.

D.2 BIOTIC AND ABIOTIC VARIABLES DEFINING THE FOOD WEB STRTUCTURE OF INLAND WATERS: LAKE ECOSYSTEM

Food web structure remains a central component of ecology due to its underlying importance in explaining patterns of diversity and ecosystem function and services (Cardinale, 2011; Thompson et al., 2012). Their relationship with the diversity, connectance among species and the stability of the ecosystems still fascinates ecologists, being the main issue of past and

present studies (e.g., Pimm, 1984; MacCann, 2000; Ricklefs, 2012; Perkins, 2013). It is known that, nowadays, food-web species are not randomly feeding across the ecosystem, but they rather use different structuring strategies, which are influenced by a range of biotic and abiotic variables (Post et al., 2000; Brin d`Amour & Dubois, 2013). A further understanding of these responses and the specific effects of each attribute is important in order to comprehend the ecology of aquatic communities (Duffy et al., 2005; Macfadyen et al., 2009; Cooper & Wissel 2012). In chapter one we used ^{15}N and ^{13}C based community-wide metric scores and food web links descriptors to assess global patterns in lake structuring and to identify main drivers defining food web strategies.

Our results found signs that, in lakes, diversity increases clustering density with species being more packed but less connected. These results are related with the hypothesis of sub-food webs which contributes with the idea that communities of greater diversity may contain tightly coupled subunits, named also functional groups, where connectance strength within these compartments declines, probably in order to remain as stable food webs (McNaughton, 1978; Moore & Hunt, 1988; Warren, 1990). Accordingly, species richness would primarily reflect diversification rather than species interactions, as reported by Ricklefs, (2012). In last term, our results would agree with limnologists, such as Wetzel (2001) and Dodds (2002), who supported the replacement of the conceptualization of the ecosystem as a linear food chain with the view that food webs are highly interconnected assemblages.

The study also explored one of the major challenges for ecologists, which is related to the prediction of how ecosystems change with environmental conditions (Jeppesen et al., 2010, 2012; Cooper & Wissel, 2012). Our results give evidences to suggest that depth, latitude, elevation and chlorophyll-*a*

content are important environmental variables influencing community structuring of lakes. Differences in ratios of littoral surface area to volume and the contribution of different primary producers to consumers may be some of the most plausible reasons explaining the differences in number of links and link density *versus* depth that we observed in our study, as has been cited in other works (Vander Zander & Vadeboncoeur, 2002; Valdeboncoeur & Vander Zander, 2002). Gradients found in food chain length and species richness related to the latitude has been also observed by Gonzalez-Bergonzoni et al. (2012) which has been largely reported as the "Theory of Latitude Diversity Gradient" (Clarke & Gaston, 2006). Although other indirect effects, such as solar and thermal radiation or temperature, may be behind the mechanisms of latitude and elevation defining food web structure of lakes, both variables can be easily used as ecological indicators. Regarding the mechanism by which the chlorophyll-*a* will be controlling the structure of lakes, we related this attribute with the well-known “trophic cascades” as internal regulations that strongly influence the way consumers are structured (Carpenter et al., 1987; Hunter & Price, 1992). Our study added a new perspective about the controls of environment on ecosystem structure, engrossing the list of environmental attributes that may have a certain degree of influence in defining the food web structure of lakes (i.e, temperature, Gonzalez-Bergonzoni et al., 2012; salinity, Cooper & Wissel, 2012; nutrient content, Carpenter et al., 1992; lake size, Takimoto & Post, 2012).

D.3 CHARACTERIZING ECOSYSTEM FUNCTIONING: N DYNAMICS FROM A MULTIDISCIPLINARY APPROACH

Fluxes and transformation of N in aquatic ecosystems has long interested ecologists, particularly because of its critical role as a limiting nutrient, and because human activities have strongly altered the N cycle (Galloway et al., 1995; Vitousek et. al., 1997; Hamilton et al., 2001). N in aquatic ecosystems is microbially transformed constantly and recycled by oxidative (i.e., nitrification, annamox) and reductive processes (i.e., denitrification, DNRA, ammonification and nitrogen fixation) within the global cycle (Herber et al., 1999). ^{15}N labeling techniques have proved enormously useful to quantify these processes simultaneously and have been widely applied to trace ammonium dynamics in streams (Peterson et al., 2001, Hall et al., 2009) and lakes (Kling et al., 1994; Hadwen et al., 2005; Epstein et al., 2012; Armengol et al., 2012). On the other hand, molecular approaches have been shown to improve our understanding of microbial ecology and N biogeochemistry through the analysis of genes encoding main metabolic N processes (Wagner and Loy 2002; Throbäck et al., 2004; Philippot & Hallin, 2005). In this section, we discuss the conclusions derived from the combination of isotope-based ^{15}N-labelled NH_4 enrichments and q-PCR measurements of the functional genes associated to the main enzymatic N pathways.

Ecological consequences on N processes in semi-arid streams influenced by agriculture practices

Human activities have altered the N dynamics in streams, mainly by increasing wastewater discharges and over-use of fertilizers, being lowland streams

located in agricultural catchments especially affected (Vitousek et al., 1997). Thus, it is important to document the ability of streams to retain and transform N in order to gain a better understanding of stream ecosystems and their relationship to water quality issues (Dodds et al., 2000). Transformation and assimilation of N in these ecosystems is complex because it involves many different processes resulting from the interaction of hydrological, chemical, and biological variables (Mulholland et al., 2000, Peterson et al., 2001; Hall and Tank, 2003).

The long processing lengths and low rates of ammonium cycling observed in Arroyo Grande del Molinillo support the idea that streams influenced by agriculture are systems poorly efficient in the retention of NH_4, contrary to forest streams which show a tight cycling and high biological demands (Newbold et al., 1981, 1982; Elwood et al., 1983; Peterson et al., 2000; Ensign and Doyle, 2006). Moreover, although nitrification rates in streams generally increase as NH_4 concentration augments (Peterson et al., 2001; Kemp and Dodds, 2002), our results from the ^{15}N addition and the abundances *Amoa A-B* functional genes give evidence to suggest a minor importance of this process pathway in streams with higher ammonium loads (Davidson et al., 1991, Stark and Hart, 1997; Mulholland et al., 2000). We related this pattern with a nonlinear response of NH_4 demands via nitrification, derived, probably from the exceeding of an ammonium concentration threshold. Similar behaviors have been reported in Kemp and Dodds (2002), Arango et al. (2008) and Dodds et al. (2010).

Regarding the assessment of other N processes, abundances of genes encoding the processes of DNRA (*nrfA*) and anammox (*16S*) in Arroyo Grande del Molinillo, indicated a marginal role of these two processes. We presume

that these rates can result, partially, because DNRA and anammox require a highly reducing (anoxic) environment (Page et al., 2003), that may not occur in the first 10 cm of the stream sediments. Anammox bacteria have been reported to live generally in aquatic ecosystems under ammonium limitation (Kartal et al., 2007), although there is still a lack of knowledge on the factors that govern these community distributions (Sonthiphand et al., 2014). Results from q-PCR measurements reported high occurrence abundances of genes encoding denitrifiyer communities (*narG, narpA, nirS and NirK*), supporting the idea that high rates of denitrification occur in the streams with high NO_3 levels, as it has been previously reported in Bernot & Dodds, (2005), Graham et al. (2010) and Mulholland et al. (2009). The removal of N by this process has been shown to reduce the export of N downstream, becoming a pivotal process that balances the flux of N into the biosphere and that can reduce the effects of excess agricultural N. The low abundance of *nosZ* genes compared with the rest of the denitrifiyers indicated a genetic capacity of the stream to potentially produce N_2O by an incomplete denitrification pathway. This could be because the presence of high NO_3 concentrations, apparently, is related to the inhibition of N_2O to N_2 conversion (Weier et al., 1993; Hefting et al., 2003). Therefore, our results support evidence that stream ecosystems, specifically low-land agricultural streams, enhance the emission of N_2O to the atmosphere, which is widely believed to affect global climate change, contributing between 6-30 % of the anthropogenic N_2O gas production (Fenchel et al., 1998; Seitzinger and Kroeze, 1998; Clough et al., 2006).

Finally, our study also remarked the importance of the hyporheic zone as a key site on nitrogen metabolism, as it has already been recognized by other authors in the past (e.g., Findlay, 1995, Boulton et al., 1998). According to this result we strongly suggest, as other authors previously (e.g. Roley et al., 2012;

Merill et al., 2014), the consideration of the hiporheic zone in restoration programs of semi-arid streams in order to provide subsurface flow increasing the total nitrification/denitrification and decreasing evapotranspiration losses.

Nitrogen dynamics in lentic systems with N- limitation

In oligotrophic lakes with short residence time and a strong influence of rapid DIN loss via outflux, often, the N is limiting the primary productivity, having important ecological consequences in the aquatic ecosystem function and structure. In this systems, submerged macrophytes can play a central role in nutrient cycling, especially in small, shallow lakes (Ozimej et al., 1993), contributing to in-lake nutrient uptake and hence, temporal N retention (Gacia et al. 1994; Marion and Paillisson 2003).

Our results from the $^{15}NH_4$ enrichment experiment remarked that submerged macrophytes showed an important demand for nitrogen, contributing to in-lake nutrient uptake, as also reported in other lakes (Gacia et al., 1994; Marion & Paillisson 2003). There are some lines of evidence that suggest that in aquatic ecosystems some species of macrophytes (i.e., *Groenlandia densa, Chara vulgaris* and *Chara hispida)* quickly incorporate the ammonium directly from the water column, indicating a preferentially short-term uptake via pelagic, instead of the benthic habitat (Best and Mantai, 1978; Ozimej et al., 1993; Touchette and Burkholder, 2000; Madsen and Cedergreen, 2002). Slow decomposition rates in these species imply that N trapped in the macrophyte tissues will be retained for a relatively long period. This behavior has been observed particularly in charophytes, which have been reported to decompose slower than other aquatic plants, prolonging nutrient storage in their biomass

(Kufel and Kufel, 2002). This clearly implies the capability of *Chara* beds to act as nutrient sinks in shallow lakes, playing an important role in nutrient cycling, particularly considering the aboveground part nutrient acquisition from the water column (Kufel and Kufel, 2002; Rodrigo et al., 2007; Siong and Asaeda, 2009). While this capacity has been related with the amount of N available in the system, the nutritional status of the cells with respect to the surrounding N and on the growing status of the aquatic plants, there are also some evidences to suggest differences in species functionality, implying diverse physiological strategies (Carignan 1982; Carr and Chambers 1998; Eugelink 1998; Vermeer et a., 2003; Lee et al. 2007). Emergent macrophytes, which are only partially covered by water, would be, presumably, less efficient in assimilating DIN from the water column. This could be because of the degree of water exposure and the structural traits of the different species (Peipoch et al., 2013). The functional attributes of different macrophytes is a crucial issue not only because they can act as one of the main mechanisms retaining N within the system, but also because they can remove excess of ammonium in high nutrient-loaded lake systems. However, the sensitiveness and vulnerability of these plants under changes in nutrient availability as occurring during eutrophication needs to be evaluated to assess resilience effects.

Beside the role of aquatic plants, nitrification has also been reported as the main mechanism controlling denitrification in lakes through coupled nitrification-denitrification process (Saunders et al., 2001; Seitzinger et al., 2006). Our results from q-PCR analyses of genes encoding main nitrifiyers (*amoA*) suggested that in oligotrophic lakes, nitrifiyers (mainly bacteria organisms, AOB) may have a key role, rapidly converting ammonium to nitrate and, hence, creating NO_3 sources which facilitate the occurrence of the

denitrification pathway, determining the distribution of DIN pools in the overall aquatic N cycle. Indeed, measurements of the abundances in denitrifiyers (*narG, napA ,nirS and nirK, nosZ*) may also evidence the potential occurrence of denitrification within the benthic and pelagic habitat of oligotrophic lakes, even though this is an apparently unsuitable environment (see Vila-Costa et al.,2014). Our results evidenced that, as expected, in oligotrophic lakes DNRA and anammox are marginal processes, mainly due to the absence of strong anoxic conditions (Page et al., 2003). However, more studies are needed to make a comprehensive analysis of the role of these apparent marginal N processes in lakes.

In both benthic and pelagic compartments, the high amount of nitrite, nitrate and nitrous oxide reductase enzyme assemblages observed may be indicating that oligotrophic lakes have a considerable capacity to complete denitrification process. That is, these lakes are effective systems for DIN removal out of the aquatic systems and are only limited by physical conditions (e.g. time retention). Our results supported the previously suggested dependence of supporting denitrifiers by coupled nitrification-denitrification mechanisms in oligotrophic lakes where NO_3 appear in low concentrations (e.g., Seitzinger, 1988; Piña-Ochoa et al., 2007; Vila Costa et al., 2014). Imbalances in the availability of ammonium/nitrate may have significance in the functioning of these oligotrophic lakes, making them systems more vulnerable to any nutrient enrichment. Improving the quality of our water bodies clearly implies promoting the N removal of N excess from agriculture, and hence, decreasing the total N exported to the oceans (Seitzinger et al., 2006; Piña-Ochoa et al., 2007). In this dissertation strong evidences supporting the importance of submerged macrophytes on the ecosystem functioning are shown, contributing to maintain a clear water status which help to increase the resilience of aquatic

ecosystems under global change effects. Disappointingly, field-based studies examining either the role of submerged macrophytes on N removal capacity of shallow lakes or their effects on microbially-mediated nitrogen transformations (e.g. nitrifiers and denitrifiers bacteria) are still scarce. Future studies using stable isotopec as tracers combined with new approaches will help these and other questions still unresolved in aquatic ecology and biogeochemistry.

E. CONCLUSIONS

CONCLUSIONES

The main conclusions from this dissertation are the following:

1. Regarding the structure and functioning of lake ecosystems, this thesis provides the following conclusions:

    a. Our results agree with the interpretation of lake structure food webs as highly interconnected assemblages, rather than as linear food chains.

    b. Lakes of greater diversity, in order to remain stable, contain species that are more packed but less connected (tightly coupled in blocks). Then, species richness would primarily reflect diversification rather than species interactions.

    c. Depth, latitude, elevation and Chlorophyll-*a* content are suggested to be some of the most important environmental drivers influencing lake community structure.

    d. In oligotrophic lakes, the occurrence of dense abundances of specific submerged macrophytes (e.g., *Groenlandia densa, Chara vulgaris* and *Chara hispida)* may have a meaningful contribution as N sinks for a relatively long period.

    e. Our results support the previously suggested dependence of denitrifiers by coupled nitrification-denitrification mechanisms in oligotrophic lakes. Imbalances in the availability of ammonium/nitrate may have significance in the functioning of these oligotrophic lakes, making them systems more vulnerable to any nutrient enrichment.

2. Regarding the main N dynamics in low-land agriculture streams, this thesis supplies the following conclusions:

a. Low-land agricultural streams may be systems poorly efficient in the retention of ammonium, with long processing lengths and low rates of nitrogen cycling.

b. In low-land agricultural streams, the threshold in the ammonium concentration may be exceeded, causing a nonlinear response and a decrease in NH_4 biological demands via nitrification.

c. In low-land agriculture streams, denitrification may be a pivotal process reducing the effects of NO_3 excess. Moreover, we suggested the potential occurrence on incomplete denitrification pathway, enhancing the emission of N_2O to the atmosphere.

d. In low-land agriculture streams, the hyporheic zone may act as a key site on nitrogen metabolism. Therefore, we strongly recommend the consideration of these areas in restoration programs of semi-arid streams.

3. Overall, from the use of ^{13}C and ^{15}N stable isotope approaches, the following conclusions arise from this thesis :

a. Stable isotopes approaches of ^{15}N and ^{13}C, through natural abundances and deliberate tracer addition, are a powerful complementary tool in the study of ecological and biogeochemical processes at whole aquatic ecosystem scale, promising remarkable progress in aquatic sciences.

b. This dissertation clearly proves that the combination of SIA, molecular approaches and other classical limnological tools,

provide a further comprehensive explanation of the N processes and their efficiencies in aquatic ecosystems.

c. We found that more investment describing the most suitable practical methodologies is needed for using $^{15}N_2$ and $^{15}N_20$ as routine measurements in *in situ* aquatic ecosystem studies.

d. We deeply encourage limnologists to take advantage of incorporating stable isotope approaches to continuously evaluate some of the main interesting ecological research questions that are still unresolved.

CONCLUSIONES

Las principales conclusiones de esta tesis son los siguientes:

1. Respecto a la estructura y funcionamiento de los ecosistemas lacustres, esta tesis ha obtenido las siguientes conclusiones:

    a. Nuestros resultados concuerdan con la interpretación de que la estructura de las redes tróficas de los lagos son conjuntos altamente interconectados, en lugar de las cadenas alimenticias lineales.
    b. En los lagos con mayor diversidad, con el fin de permanecer estables las especies están más empaquetadas (fuertemente acopladas en bloques). pero menos interconectadas; así, la riqueza de especies refleja más una diversificación que una mayor interacción trófica entre especies.
    c. La profundidad, la latitud, la elevación y la clorofila-a se muestran como los factores ambientales más importantes que influyen en la estructura de las comunidades de los lagos.
    d. En los lagos oligotróficos, la ocurrencia de densas praderas de macrófitos sumergidos (por ejemplo, *Groenlandia densa, Chara vulgaris* y *Chara hispida*) contribuye significativamente al papel de sumidero de N de estos ecosistemas en periodos de tiempo relativamente largos.
    e. Nuestros resultados confirman la dependencia en lagos oligotróficos de los desnitrificadores de los mecanismos acoplados de nitrificación-desnitrificación. Cualquier desequilibrio en la disponibilidad de amonio o nitrato pueden

tener importancia en el funcionamiento de estos lagos, lo que hace a estos sistemas más vulnerables a cualquier enriquecimiento de nutrientes.

2. Respecto a la dinámica del N los arroyos influenciados por la agricultura, esta tesis suministra las siguientes conclusiones:

a. Este tipo de sistemas fluviales son muy poco eficientes en la retención y transformación de amonio, con longitudes de procesamiento largas y bajas tasas de transformación del nitrógeno.

b. En estos arroyos agrícolas se puede estar excediendo un umbral de concentración de amonio que está causando una respuesta no lineal y una disminución en las demandas biológicas de NH_4 para la nitrificación.

c. En este tipo de cursos de agua, la desnitrificación es un proceso fundamental para reducir los efectos de exceso de NO_3 aportado por la agricultura. Sin embargo, nuestros resultados sugieren la aparición de una desnitrificación incompleta que estaría aumentado las emisiones de N_2O a la atmósfera.

d. El hiporreos es un lugar clave en el metabolismo del nitrógeno en éste tipo de arroyos, por lo tanto, es fundamental considerarlo como parte integral en los proyectos de restauración de los sistemas fluviales semiáridos.

3. Por último, en términos generales desde el uso los isótopos estables de ^{13}C y ^{15}N como trazadores en ecología y biogeoquímica acuática, de esta tesis se desprenden las siguientes conclusiones:

a. Los isótopos estables de ^{15}N y ^{13}C tanto a través de la determinación de sus abundancias naturales como de su adición deliberada, son una poderosa herramienta complementaria para el estudio de los procesos ecológicos y biogeoquímicos a escala de ecosistema, prometiendo notables progresos en las ciencias acuáticas.

b. En esta tesis se demuestra claramente que la combinación de SIA con los estudios moleculares y con otras herramientas clásicas usadas en limnología, mejoran las interpretaciones y predicciones sobre el funcionamiento de los procesos asociados al ciclo del N en los ecosistemas acuáticos.

c. Es necesario mejorar en las metodologías para determinar los cambios en las firmas isotópicas de los gases ($^{15}N_2$ y $^{15}N_2O$) para aprovechar mejor su potencias en estudios a escala de ecosistema.

d. Por último, este trabajo sirve para animar a muchos de los investigadores dedicados a la limnología y biogeoquímica a que incorporen el enfoque de los isótopos estables puesto que puede ayudar a resolver algunos de los paradigmas vigentes en diferentes campos de la ecológica acuática.

REFERENCES

Abrantes, K.G., Barnett, A., Marwick, T.R. Bouillon, S. 2013. Importance of terrestrial subsidies for estuarine food webs in contrasting east African catchments. Ecosphere: 4 (1), 1-33.

Abrantes, K.G., Barnett, A., Bouillon, S. 2014. Stable isotope-based community metrics as a tool to identify patterns in food web structure in east African estuaries. Functional Ecology: 28, 270–282.

Acuña, V., Gausch H., Giorgi A., Izagirre O. 2009. "Flujo de energía en el ecosistema". En Conceptos y técnicas en ecología fluvial. Elosegi A. y Sabater S. Ed. Bilbao.

Alexander, R.B., Smith, R.A., Schwarz, G.E. 2000. Effect of stream channel size on the delivery of nitrogen to the Gulf of Mexico. Nature 403: 758–761.

Amber, A.K. 2009. Interactions between macroalgae and the sediment microbial community: Nutrient cycling within shallow coastal bays. PhD Dissertation, The Faculty of the School of Marine Science, Virginia Institute of Marine Sciences.

Amber, A.K., Canuel, E.A., Anderson, I.C., Veuger, B. 2010. Fate of macroalgae in benthic systems: carbon and nitrogen cycling within the microbial community. Mar Ecol Prog Ser 414:41-55.

An, S. and Gardner, W.S. 2002. Dissimilatory nitrate reduction to ammonium (DNRA) as a nitrogen link, versus denitrification as a sink in a shallow estuary (Laguna Madre/Baffin Bay, Texas). Marine Ecology Progress Series: 237, 41–50.

Anderson T.K., Sukhdeo M.V.K. 2011. Host Centrality in Food Web Networks Determines Parasite Diversity. PloS One: 6 (10), e26798.

APHA. 1995. Standard Methods for the Examination of Water and Wastewater, 19th edn. American Public Health Association, Washington, DC.

Arango C.P., Tank J.L., Johnson L.T., Hamilton S.K. 2008. Assimilatory uptake rather than nitrification and denitrification determines nitrogen removal patterns in streams of varying land use. Limnology and Oceanography, 53, 2558–2572.

Arcagnia, M., Campbellc L.M., Arribérea M.A., KurtKysere D., Klassene K., Casauxf R., Miserendinof M.L., Guevara S.R. 2013. Food web structure in a double-basin ultra-oligotrophic lake in Northwest Patagonia,

Argentina, using carbon and nitrogen stable isotopes. Limnologica: 43 (2), 131–142.

Armengol, X., Wurtsbaugh, W.A., Camacho, A., Miracle, M.R. 2012. Pseudo-diel vertical migration in zooplankton: a whole-lake ^{15}N tracer experiment. Journal of Plankton Research: 1-12.

Armstrong, R.A and McGehee, R. 1980. Competitive exclusion. American Naturalist: (115), 151-170.

Arp, C. D., Gooseff, M.N., Baker, M.A., Wurtsbaugh, W. 2006. Surface-water hydrodynamics and regimes of a small mountain stream–lake ecosystem. J. Hydrol. 329: 500–513.

Arrigo, K.R. 2005. Review Marine microorganisms and global nutrient cycles. Nature: 437, 349-355.

Ashkenas, L.R., S.L. Johnson, S.V. Gregory, J.L. Tank, and W.M. Wollheim. 2004. A stable isotope tracer study of nitrogen uptake and transformation in an old-growth forest stream. Ecology 85:1725-1739.

Auerbach, M.J. 1984. Stability, probability and the topology of food webs. In: Strong DR, Simberloff D, Abele LG, Thistle AB (eds) Ecological Communities, Conceptual Issues and the Evidence, Princetown University Press, Princetown, N.J., pp 413–436.

Auguet, J.C., Nomokonova, N., Camarero, L., Casamayor, E.O. 2011. Seasonal changes of freshwater ammonia-oxidizing archaeal assemblages and nitrogen species in oligotrophic alpine lakes. Applied and Environmental Microbiology: 77, 1937–1945.

Auguet, J.C., Triado-Margarit, X., Nomokonova, N., Camarero, L., Casamayor, E.O. 2012. Vertical segregation and phylogenetic characterization of ammonia-oxidizing Archaea in a deep oligotrophic lake. Multidisciplinary Journal of Microbial Ecology: 6, 1786–1797.

Aumen, N. G. 1990. Concepts and methods for assessing solute dynamics in stream ecosystems. J. N. Am. Benthol. Soc. 9: 95–119.

Barko, J. W., Gunnison, D., Carpenter, S.R. 1991. Sediment interactions with submersed macrophyte growth and community dynamics. Aquat. Bot. 41: 41–65.

Bartels, P., Cucherousset, J., Gudasz, C., Jansson, M., Karlsson, J., Persson, L., Premke, K., Rubach, A., Steger, K., Tranvik, L.J. 2012. Terrestrial subsidies to lake food webs: an experimental approach. Oecologia 168:807–818.

Bartrons, M., Camarero, L., Catalan, J. 2010. Isotopic composition of dissolved inorganic nitrogen in high mountain lakes: variation with altitude in the Pyrenees. Biogeosciences: 7, 1469–1479.

Bearhop, S., Adams, C.E., Waldron, S., Fuller, R.A., Macleod, H. 2004. Determining trophic niche width: a novel approach using stable isotope analysis. Journal of Animal Ecology: 73, 1007–12.

Bellwood, D. R., Hughes, T.P., FOLKE, C., Nystrom, M. 2004. Confronting the coral reef crisis. Nature 429:827–833.

Benstead, J.P., March, J.G., Fry, B., Ewel, K.C., Pringle, C.M. 2006. Testing isosource: Stable Isotope Analysis of a tropical fishery diverse organic matter sources. Ecology 87:326–333.

Bernot, M.J. and Dodds, W.K. 2005. Nitrogen retention, removal, and saturation in lotic ecosystems. Ecosystems 8: 442–53.

Bernot, M.J., Dodds, W.K., Gardner, W.S., McCarthy, M.J., Sobolev, D., Tank J.L. 2003. Comparing denitrification estimates for a Texas estuary by using acetylene inhibition and membrane inlet mass spectrometry. Appl. Environ. Microbiol. 69: 5950–595.

Bersier, L.F., Banasek-Richter, C., Cattin, M.F. 2002. Quantitative descriptors of food web matrices. Ecology: 83, 2394–2407.

Billen, G., Lancelot, C., Meybeck, M. 1991. N, P, and Si retention along the aquatic continuum from land to ocean. In: Matoura RFC, Martin JM & Wollast R (Ed) Ocean Margin Processes in Global Change (pp 19–44). Wiley and Sons, Chichester.

Boschker HTS, Middleburg JJ (2002) Stable isotopes and biomarkers in microbial ecology. FEMS Microbiol Ecol 40:85–95

Bouillon, S., Gillikin, D. P., and Connolly, R. M. 2012. Use of stable isotopes to understand food webs and ecosystem functioning in estuaries, in: Treatise on Estuarine and Coastal Science, edited by: Wolanski, E. and McLusky, D. S., 7, 143–173, Waltham: Academic Press.

Boulton, A.J., Findlay, S., Marmonier, P., Stanley, E.H. and Valett, H.M. 1998. The functional significance of the hyporheic zone in streams and rivers. Annual Review of Ecology and Systematics, 29, 59-81.

Boulêtreau, S., Lyautey, E., Dubois, S., Compin, A., Delattre, C., Touron-Bodilis, A., Mastrorillo, S., Garabetian, F. 2014. Warming-induced changes in denitrifier community structure modulate the ability of

phototrophic river biofilms to denitrify. Science of The Total Environment: 467, 856–863.

Bowden WB, Peterson BJ, Hobbie JE, Steudler PA, Moore, B. 1991. Transport and processing of nitrogen in a tidal freshwater wetland. Wat Res Res 27:389–408.

Boyer, E.W., Goodale, C.L, Jaworski, N.A, Howarth, R.W. 2002. Anthropogenic nitrogen sources and relationships to riverine nitrogen export in the northeastern U.S.A. Biogeochemistry: 57/58, 137–169.

Böhlke, J.K., Harvey, J.W., Voytek, M.A. 2004. Reach-scale isotope tracer experiment to quantify denitrification and related processes in a nitrate-rich stream, midcontinent United States. Limnology and Oceanography 49:821-838.

Böhlke, J.K., Antweiler, R.C., Harvey, J.W., Laursen, A.E., Smith, L.K., Smith, R.L., Voytek, M.A. 2009. Multi-scale measurements and modeling of denitrification in streams with varying flow and nitrate concentration in the upper Mississippi River basin, USA. Biogeochemistry 93:117–141.

Brandes, J. A., McClain, M.E., Pimentel, T.O. 1996. N-15 evidence for the origin and cycling of inorganic nitrogen in a small Amazonian catchment. Biogeochemistry, 34(1), 45–56.

Bratkič, A., Šturm, M., Faganeli, J., Ogrinic, N. 2012. Semi-annual carbon and nitrogen isotope variations in the water column of Lake Bled (NW Slovenia) Biogeosciences, 9, 1-11.

Briand, F. 1983. Environmental-control of food web structure. Ecology: 64 (2), 253-263.

Brind'Amour, A. and Dubois, S.F. 2013. Isotopic Diversity Indices: How Sensitive to Food Web Structure? PLoS ONE: 8(31), e84198.

Brookes, P.C., Landman, A., Pruden, G. y Jenkinson, D.S. 1985. Chloroform fumigation and the release of soil nitrogen: a rapid direct extraction method to measure microbial biomass nitrogen in soil. Soil Biology & Biochemistry 17(4): 837-842.

Bru, D., Sarr, A., Philippot, L., 2007. Relative abundances of proteobacterial membrane-bound and periplasmic nitrate reductases in selected environments. Applied and Environmental Microbiology: 73 (18), 5971e5974.

Bryant, J.A., Lamanna, C., Helene, H., Kerkhoff, A.J., Enquist, B.J., Green, J.L. 2008. Microbes on mountainsides: Contrasting elevational patterns of bacterial and plant diversity. PNAS: 104 (1), 11505-11511.

Bull, I.D., Parekh, N.R., Hall, G.H., Ineson, P., Evershed, R.P. 2000. Detection and classification of atmospheric methane oxidizing bacteria in soil. Nature 405:175–178.

Burgin, A.J. and Hamilton, S.K. 2007. Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Frontiers in Ecology and the Environment: 5, 89–96.

Burgin, A.J., Hamilton, S.K., Jones, S.E., Lennon, J.T. 2012. Denitrification by sulphur-oxidizing bacteria in a eutrophic lake. Aquatic Microbial Ecology: 66, 283-293.

Cabana, G. and Rasmussen, J. B. 1996. Comparison of aquatic food chains using nitrogen isotopes. Proceedings of the National Academy of Sicence, USA: 93, 10844–10847.

Cardinale, B.J. 2011. Biodiversity improves water quality through niche partitioning. Nature: 472, 86–89.

Carlson, R.E. 1977. A trophic state index for lakes. Limnology and Oceanography: 22,(2) 361-369.

Carman, K.R., Fry B. 2002. Small-sample methods for $\delta^{13}C$ and $\delta^{15}N$ analysis of the diets of marsh meiofaunal species using atural abundance and tracer-addition isotope techniques. Mar Ecol Prog Ser 240:85-92

Carpenter, S.R., Kitchell, J.F., and Hodgson, J.R. 1985. Cascading trophic interactions and lake productivity. BioScience: 35, 634-39.

Carpenter, S.R., Kitchell J.F., Hodgson J.R., Cochran P.A., Elser, J.J., Elser, M.M., Lodge, D.M., Kretchmer D., He, H., and C.N. Von Ende, C.N. 1987. Regulation of lake primary productivity by food web structure. Ecology 68: 1863-76.

Carpenter, S.R., Cottingham, K.L., Schindle, D.E. 1992. Biotic feedbacks in Lake phosphorus cycles. Review Article. Trends in Ecology and Evolution: 7 (10), 332-336.

Carpenter, S,R,, Cole, J.J., Pace, M.L., Van de Bogert, M., Bade, D.L., Bastviken, D., Gille, C.M., Hodgson, J.R., Kitchell, J.F., Kritzberg, E.S.2005. Ecosystem subsidies: terrestrial support of aquatic food webs from ^{13}C addition to contrasting lakes. Ecology: 86,2737-2750.

Carpenter, S.R., Stanley, E.H., Vander Zanden, M.J. 2011. State of the world's freshwater ecosystems: physical, chemical, and biological changes. Annual Review of Environment and Resources: 36 (1), 75–99.

Catalan, J., Camarero, L., Gacia, E., Ballesteros, E., Felip, M. 1994. Nitrogen in the Pyrenean lakes (Spain). Hydrobiologia: 274, 17–27.

Christensen, P.B., Glud, R.N., Dalsgaard, T., Gillespie, P. 2003. Impacts of longline mussel farming on oxygen and nitrogen dynamics and biological communities of coastal sediments. Aquaculture: 218 (1–4), 567-588.

Cheneby, D., Philippot, L., Hartmann, A., Henault, C.F., Germon, J.C., 2000. 16S rRNA analysis for characterization of denitrifying bacteria isolated from three agricultural soils. FEMS Microbiology Ecology: 34, 121–128.

Chon, K., Chang, J.S., Lee, E., Lee, J., Ryu, J., Cho, J., 2011. Abundance of denitrifying genes coding for nitrate (nar G), nitrite (nir S), and nitrous oxide (nos Z) reductases in estuarine versus wastewater effluent-fed constructed wetlands. Ecological Engineering: 37, 64–69.

Cifuentes, L.A., Sharp, J.H., Foger, M.L 1988. Stable carbon and nitrogen isotope biogeochemistry in the Delaware Estuary. Limnology and Oceanography: 33, 1102-1115.

Clarke, A. and Gaston K.J. 2006. Climate, energy and diversity. Proceedings of the Royal Society of London B: 273, 2257–2266.

Clayton,D. 2003. Handbook of Isotopes in the Cosmos. Cambridge University Press, New York.

Cole, J.J., Pace, M.L., Carpenter, S.R., Kitchell, J.F. 2000. Persistence of net heterotrophy in lakes during nutrient addition and food web manipulation. Limnology and Oceanographt: 45,1718–1730.

Cole, J.J., Carpenter, S.R., Kitchell, J.F., Pace, M.L. 2002. Pathways of organic carbon utilization in small lakes: results from a whole-lake C13 addition and couple model. Limnology and Oceanographt: 47,1664-1675.

Cole, J.J., Carpenter, S.R., Pace, M.L., Van de Bogert, M.C., Kitchell, J.L., Hodgson, J.R. 2006. Differential support of lake food webs by three types of terrestrial organic carbon. Ecology Letters: 9, 558–568.

Connan, M., McQuaid C.D., Bonnevie B.T., Smale M.J., Cherel. Y. 2014.Combined stomach content, lipid and stable isotope analyses reveal spatial and trophic partitioning among three sympatric albatrosses from the Southern Ocean. Marine Ecology Progress Series: 497, 259-272.

Correa-Galeote, D., Diana E., Tortosa M.G., Bru, D., Philippot, L., Bedmar, E.J. 2013. Spatial distribution of N-cycling microbial communities showed complex patterns in constructed wetland sediments. FEMS Microbiology Ecology: 83, 340–351.

Cosgrove, W.J. and Rijsberman, F.R. 2014. *World water vision: making water everybody's business*. Earthscan, London.

Cooper, R.N. and Wissel, B. 2012. Loss of trophic complexity in saline prairie lakes as indicated by stable-isotope based community metrics. Aquatic Biosystems: 8 (6).

Craig, Harmon.1953, The geochemistry of the stable carbon isotopes: Geochim et Cos- v 3, p 53-92.

Dahm, C.N., Valett, H.M., Baxter, C.V., Woessner, W.W. 2006. Hyporheic zones. In: Methods in Stream Ecology (Eds F.R. Hauer & G.A. Lamberti), pp. 119–142. Elsevier, Boston.

Dale, O., Tobias, C., Song, B. 2009. Biogeographical distribution of diverse anaerobic ammonium oxidizing (anammox) bacteria in Cape Fear River Estuary. Environmental Microbiology: 11, 1194–1207.

Davidson, E. A., Hart, S. C., Shanks, C. A., Firestone, M. K. 199. Measuring gross nitrogen mineralization, immobilization, and nitrification by 15N isotopic pool dilution in intact soil cores. Journal of Soil Science: 42, 335-349.

Davis, J. C. and Minshall, G.W. 1999. Nitrogen and phosphorus uptake in two Idaho (USA) headwater wilderness streams. Oecologia: 119, 247–255.

D'Angelo, D.J., Webster, J.R., 1991. Phosphorus retention in streams draining pine and hardwood catchments in the southern Appalachian mountains. Freshwater Biology: 26, 335–345.

De'Ath, G. and Fabricius K.E. 2000. Classification and regression trees: a powerful yet simple technique for ecological data analysis. Ecology : 81 (11), 3178-3192.

De'Ath, G. 2002. Multivariate regression trees: a new technique for modeling species environment relationships. Ecology: 83, 1105-1117

De Beer, D., and Schramm, A. 1999. Microenvironments and mass transfer phenomena in biofilms studied with microsensors. Water Science and Technology: 39, 173-178.

DeNiro, M.J. and Epstein, S. 1976. You are what you eat (plus a few permil): the carbon isotope cycle in food chains. Geological Society of America Abstracts with Programs: 8, 834–835.

DeNiro, M.J. and Epstein, S. 1978. Influence of diet on the distribution of carbon isotopes in animals. Geochimica et Cosmochimica Acta: 42, 495–506.

DeNiro, M.J. and Epstein, S. 1981. Influence of diet on the distribution of nitrogen isotopes in372 animals. Geochimica et Cosmochimica Acta 45:341-351.

Dodds, W.K. and Jones, R.D. 1987. Potential rates of nitrification and denitrification in an oligotrophic freshwater sediment system. Microbial Ecology 14:91–100.

Dodds, W.K. 2002. Freshwater ecology: Concepts and environmental applications. Academic Press, San Diego. 569 p.

Dodds, W.K., M.A. Evans-White, N. Gerlanc, L. Gray, D. A. Gudder, M.J. Kemp, A.L. Lopez, D. Stagliano, E. Strauss, J.L. Tank, M.R. Whiles, and W. Wollheim. 2000. Quantification of the nitrogen cycle in a prairie stream. Ecosystems: 3, 574-589.

Dodds, W.K., López, A., Bowden, W.B., Gregory, S., Grimm, N.B., Hamilton, S.K., Hershey, A.E., Martí, E., McDowell, W.H., Meyer, J.L. 2002. N uptake as a function of concentration in streams. Journal of the North American Benthological Society: 21, 206-220.

Doi, H., Kikuchi, E., Shikano, S., Takagi, S.A. 2004. Study of the nitrogen stable isotope dynamics of phytoplakton in a simple natural ecosystem. Aquatic microbial ecology: 36, 285-291.

Drake, D.C., Peterson, B.J., Galván, K., Deegan, L.A., Hopkinson, C., Johnson, J.M., Koop-Jakobsen, K., Lemay, L.E., Picard, C. 2009. Saltmarsh ecosystem biogeochemical responses to nutrient enrichment: a paired ^{15}N tracer study. Ecology: 90, 2535-2546.

Duff, J. H., and F. J. Triska. 1990. Denitrification in sediments from the hyporheic zone adjacent to a small forested stream. Canadian Journal of Fisheries and Aquatic Sciences: 47, 1140–1147.

Duff, J.H., Triska, F.J. 2000. Nitrogen biogeochemistry and surface-subsurface exchange in streams. In: Jones JB, Mulholland PJ (eds) Streams and ground waters. Academic Press, San Diego.

Duffy, E.J., Paul Richardson, J., France, K.E. 2005. Ecosystem consequences of diversity depend on food chain length in estuarine vegetation. Ecology Letters: 8, 301–309.

Dunson, W.A. and Travis, J. 1991. The role of abiotic factors in community organization. American Naturalist: 138(5), 1067-1091.

Dumont, M.G., Murrell, J.C. 2005. Stable isotope probing- linking microbial identity to function. Nature Review Microbiology: 3, 499-504.

Earl, S. R., Valett, H. M., Webster, J. R. 2006. Nitrogen saturation in stream ecosystems. Ecology: 87, 3140–3151.

Elosegi, S. and Butturini, A. 2009. El transporte de los materiales inorgánicos disueltos y particulados. En: Conceptos y técnicas en ecología fluvial, Elosegi, A. y Sabater, F. (Eds), pp. 83-96. Fundación BBVA. Bilbao

Elser, J.J., Bracken, M.E.S., Cleland, E.E., Gruner, D.S., Harpole, W.S., Hillebrand, H. et al. 2007. Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. Ecology Letters: 10, 1135– 1142.

Ensign, S.H. and Doyle, M.W. 2006. Nutrient spiraling in streams and river networks. Biogeosciences: 111, G04009.

Elwood, J.W., Newbold, J.D., O´Neill and Van Winkle W. 1983. Resource spiraling: an operational paradigm for analyzing lotic ecosystems. Dynamics of lotic ecosystems. Ann Arbor Science: 3-27.

Epstein, D.M., Wurtsbaugh, W.A., Baker, M. A. 2012. Nitrogen Partitioning and Transport Through a Subalpine Lake Measured with an Isotope Tracer. Master thesis. Watershed Sciences Faculty Publications.

Erhardt, E.B., Wolf, B.O., Ben-David, M., Bedrick, E.J. 2014. Stable Isotope Sourcing using Sampling. Open Journal of Ecology: 4(6), 289–298.

Eyre BD (2000) Regional evaluation of nutrient transformation and phytoplankton growth in river-dominated sub-tropical east Australian estuaries. Marine Ecology Progress Series: 205, 61-83.

Falkowski, P.G. 1997. Evolution of the nitrogen cycle and its influence on the biological 1422 sequestration of CO2 in the ocean. Nature: 387 (6630), 272-275.

Fath, B.D., Scharlerb, U.M., Ulanowiczd, R.E., Hannone, B. 2007. Ecological network analysis: network construction. Ecology modelling: 208, 49–55.

Findlay, S., P. Mulholland, S. Hamilton, J. Tank, M. Bernot, A. Burgin, C. Crenshaw,W. Dodds, N. Grimm, W. Mcdowell, J. Potter, and D. Sobota

2011. Crossstream comparison of substrate-specific denitrification potential. Biogeochemistry: 104, 381-392.

Fogel, M.L. and Cifuentes, L.A. 1993. Isotope fractionation during primary production. In Engel, M.H., Macko, S.A. eds. Organic geochemistry. Plenum Press. New York. 73-98.

Francis, C.A., Beman, J.M., Kuypers, M.M. 2007. New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. Multidisciplinary Journal of Microbial Ecology: 1, 19–27.

Fry, B. 1991. Stable isotope diagrams of freshwater food webs. Ecology*:* 72, 2293–2297.

Fry, B. 2000. Using state isotopes to monitor watershed influences on aquatic trophodynamics. Canadian Journal of Fisheries and Aquatic Sciences: 56, 2167–2171.

Fry, B., Baltz, D.M., Benfield, M.C., Fleeger, J.W., Gace, A., Hass, H.L., Quiñones-Rivera, Z.J. 2003. Stable isotope indicators of movement and residency for Brown Shrimp (*Farfantepenaeus aztecus*) in Coastal Louisiana Marshscapes. Estuaries 26:82-97.

Fry, B. 2006. Stable Isotope Ecology. New York: Springer 308pp.

Galván, K., Fleeger, J.W., Fry, B. 2008. Stable isotope addition reveals dietary importance of phytoplankton and microphytobenthos to saltmarsh infauna. Marine Ecology Progress Series: 359, 37-49.

Galván, K., Fleeger, J.W., Peterson, B., Drake, D., Deegan, L.A., Johnson, D.S. 2012. Natural abundance stable isotopes and dual isotope tracer additions help to resolve resources supporting a saltmarsh food web. Journal of Experimental Marine Biology and Ecology: 410, 1-11.

Galloway, J. N., dentener, F.J., Capone, D.G., Boyer, E.W., Howarth, R.W., Seitzinger, S.P., Asner, G.P., Clveland, C.C., Green, P.A., Holland, E.A., et al. 2004. Nitrogen cycles: Past, present and future, Biogeochemistry, 70, 153–226.

Gardner, M.R. and Ashby, W.R., 1970. Connectance of large, dynamical (cybernetic) systems. Nature: 228, 784.

García, A.M., Hoeinghaus, D.J, Vieira, J.P., Winemiller, K.O. 2007. Isotopic variation of fishes in freshwater and estuarine zones of a large subtropical coastal lagoon. Estuary, Coastal and Shelf Science: 73, 399-408.

García-Lledo´ A., Vilar-Sanz, A., Trias, R., Hallin, S., Bañeras, L. 2011. Genetic potential for N2O emissions from the sediment of a free water surface constructed wetland. Water Research: 45, 5621-5632.

Garnier, J., P. Servais, G. Billen, M. Akopian, and N. Brion. 2001. The oxygen budget in the Seine estuary: balance between photosynthesis and degradation of organic matter. Estuaries 24:964–977.

Geets, J., de Cooman, M. Wittebolle, L., Heylen, K., Vanparys, B., De Vos, P., Verstraete, W., Boon, N. 2007. Real-time PCR assay for the simultaneous quantification of nitrifying and denitrifying bacteria in activated sludge. Applied Microbiology and Biotechnology: 75, 211–221.

Glibert, P.M., Fullerton, D,F., Burkholder, J.M., Cornwell, J.C., Kana, T.M. 2011. Ecological Stoichiometry, Biogeochemical Cycling, Invasive Species, and Aquatic Food Webs: San Francisco Estuary and Comparative Systems. Reviews in Fisheries Science: 19 (4), 358-417.

Goldman, C.R. M. Kumagari and R.D. Robarts (eds.). 2013. Climatic change and global warming of inland waters: impacts and mitigation for ecosystems and societies. John Wiley and Son Ltd, UK.

González-Bergonzoni, L., Meerhoff, M., Davidson, T., Teixeira-de Mello, F., Baattrup-Pedersen, A., Jeppesen, E. 2012. Meta analysis shows a consistent and strong latitudinal pattern in fish omnivory across ecosystems. Ecosystems: 15,492-503.

Grace, M. and Imberger, S. 2006. Stream Metabolism: Performing and Interpreting Measurements, Water Studies Centre Monash University, Murray Darling Basin Commission and New South Wales Department of Environment and Climate Change.

Grahama, D.W., Clare Trippett, C., Dodds, W.K., O'Brien J.M., Banner E.B.K., Head, I.M., Smith M.S., Yanga, R.K, Knappa, C.W. 2010. Correlations between in situ denitrification activity and nir-gene abundances in pristine and impacted prairie streams. Environmental Pollution: 158(10), 3225–3229.

Grey, J. and Jackson, M.C. 2012. Leaves and Eats Shoots: Direct Terrestrial Feeding Can Supplement Invasive Red Swamp Crayfish in Times of Need. PLoS. ONE: 7(8). e42575.

Grey, J., Thackeray, S.J, Jones, R.I., Shine, A. 2002. Ferox Trout (Salmotrutta) as 'Russian dolls': complementary gut content and stable isotope

analyses of the Loch Ness food web. Freshwater Biology: 47, 1235–1243.

Gribsholt, B. and Boschker, H.T.S. 2005. Nitrogen processing in a tidal freshwater marsh: A whole ecosystem ^{15}N labeling study. Limnology and Oceanography: 50, 1945-1959.

Gribsholt, B., Struyf, E., Tramper, A., Andersson, M.G.I, Brion, N., De Brabandere, L., Van Damme, S., Meire, P., Middelburg, J.J., et al. 2006 Ammonium transformation in a nitrogen-rich tidal freshwater marsh. Biogeochemistry 80:289–298.

Gribsholt, B,, Struyf, E., Tramper, A., De bradandere, L., Brion, N., Van Damme, S., Meire, P., Dehairs, F., Middleburg, J.J., Boschker, H.T.S. 2007. Nitrogen assimilation and short term retention in a nutrient-rich tidal freshwater marsh- a whole ecosystem ^{15}N enrichment study. Biogeosciences 4:11-26.

Gribsholt, B., Veuger, B., Tramper, A., Middelburg, J.J., Boschker, H.T.S. 2009. Long-term ^{15}N-nitrogen retention in tidal freshwater marsh sediment: elucidating the microbial contribution. Limnology and Oceanography: 54, 13–22.

Grimm, N.B. and Fisher, S.G. 1986. Nitrogen limitation in a Sonoran Desert stream. Journal of the North American Benthological Society: 5, 2-15.

Grimm, N.B., Arrowsmith, J.R., Eisinger, C., et al. 2004. Effects of urbanization on nutrient biogeochemistry of aridland streams. In: DeFries R, Asner GP, and Houghton R (Eds). Ecosystem interactions with land-use change. Washington, DC: American Geophysical Union.

Hadas, O., Altabet, M. A., and Agnihitori, R. 2009. Seasonally varying nitrogen isotope biogeochemistry of particulate organic matter in lake Kinneret, Israel, Limnology and Oceanography: 54, 75–85.

Hadwen, W.L. and Bunn, S.E. 2005a. Food web responses to low-level nutrient and ^{15}N-tracer additions in the littoral zone of an oligotrophic dune lake. Limnology and Oceanography: 50, 1096-1105.

Hadwen, W. L. and Bunn, S.E. 2005b. Within-lake detection of the effects of tourist activities 881 in the littoral zone of oligotrophic dune lakes. Aquatic Ecosystem Health and Management: 8, 159–173.

Hall, G. H. 1984. Measurement of nitrification rates in lake sediments: Comparison of the nitrification inhibitors nitrapyrin and allylthiourea. Microbial Ecology: 10, 25-36.

Hall, R.O. 1995. Use of a stable carbon isotope addition to trace bacterial carbon through a stream food web. Journal of the North American Benthologial Society: 14, 269-277.

Hall, R.O., Peterson, B.J., Meyer, J.L. 1998. Testing a nitrogen-cycling model of a forest stream by using a ^{15}N tracer addition. Ecosystems: 1, 283–298.

Hall, R.O. and Meyer, J.L. 1998. The trophic significance of bacteria in a detritus-based stream food web. Ecology: 79, 1995-2012.

Hall, R.O., Wallace, J.B., Eggert, S.L. 2000. Organic matter flow in stream food webs with reduced detrital resource base. Ecology: 81,3445-3463.

Hall, R. O., and Tank, J.L. 2003. Ecosystem metabolism controls nitrogen uptake in streams in Grand Teton National Park, Wyoming. Limnology and Oceanography: 48, 1120–1128.

Hall, R.O., Baker, M.A., Arp, C.D., Koch, B.J. 2009a. Hydrologic control of nitrogen removal, storage, and export in a mountain stream. Limnology and Oceanography: 54, 2128-2142.

Hall, R.O., Tank, J.L., Sobota, D.J., Mulholland, J.P., O'Brien, J.M., Dodds, W.K., Webster, J.R., Valett, H.M., Poole, G.C., Peterson, B.J., and others. 2009b. Nitrate removal in stream ecosystems measured by ^{15}N addition experiments: total uptake. Limnology and Oceanography: 54, 653-665.

Hallin, S., Jones, C.M., Schloter, M., Philippot, L., 2009. Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. The ISME Journal: 3 (5), 597e605.

Hamilton, S.K., Tank, J.L., Raikow, D.F., Wollheim, W.M., Peterson B.J., Webster, J.R. 2001. Nitrogen uptake and transformation in a midwestern US stream: A stable isotope enrichment study. Biogeochemistry: 54, 297-340.

Hamilton, S.K., Tank, J.L., Raikow, D.F., Siler, E.R., Dorn, N.J., Leonard, N.E. 2004. The role of instream vs allochthonous N in stream food webs: modeling results of an isotope addition experiment. Journal of the North American Benthological Society: 23, 429-448.

Hamilton, S.K. and Ostrom, N.E., 2007. Measurement of the stable isotope ratio of dissolved N2 in 15N tracer experiments. Limnology and Oceanograpgy: 5, 233e240.

Hamersley, M.R., Woebken, D., Boehrer, B., Schultze, M., Lavik, G.,Kuypers, M. 2009. Water column anammox and denitrification in a temperate

permanently stratified lake (Lake Rassnitzer, Germany). Systematic and Applied Microbiology: 32, 571–582.

He, J., Shen, J., Zhang, L., Zhu, Y., Zheng, Y., Xu, M., and Di, H.J. 2007. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammoniaoxidizing archaea of a Chinese upland red soil under long-term fertilization practices. Environmental Microbiology: 9, 2364– 2374.

Henry, S., Bru, D., Stres, B., Hallet, S., Philippot, L., 2006. Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. Applied and Environmental Microbiology: 72(8), 5181e5189.

Herbert, R.A. 1999. Nitrogen cycling in coastal marine ecosystems. FEMS Microbiology Reviews: 23, 563–590.

Hobbie, E.A., Macko, S.A., Shugart, H.H. 1999. Interpretation of nitrogen isotope signatures using NIFTE model. Oecologia: 120, 405-415.

Hoeinghaus, D.J. and Zeug, S.C. 2008. Can stable isotope ratios provide for community-wide measurements of trophic structure? Comment. Ecology: 89, 2353–2357.

Holmes, R.M., McClelland, J.W., Sigman, D.M., Fry, B., Peterson, B.J. 1998. Measuring ^{15}N-NH_4 in marine, estuarine and fresh waters: An adaptation of the ammonia diffusion method for samples with low ammonium concentrations. Marine Chemistry: 60, 235–243.

Holmes, R.M., Peterson, B.J., Deegan, L.A., Hughes, J.E., Fry, B. Nitrogen biogeochemistry in the oligohaline Zone of a New England Estuary. 2000. Ecology: 81 (2), 416-432.

Holtgrieve, G.W., Schindler, D.E., Branch, T.A., A'mar, Z.T. 2010. Simultaneous quantification of aquatic ecosystem metabolism and reaeration using a Bayesian statistical model of oxygen dynamics. Limnology and Oceanography: 55,1047–1063.

Howarth, R. W., Billen, G., Swaney, A., Townsend, N., Jaworski, N., Lajtha, K., Dorning, J.A., Elmgren, N., Caraco, T., Jordan, F., and others. 1996. Regional nitrogen budgets and riverine N & P fluxes for the drainages to the North Atlantic Ocean: natural and human influences. Biogeochemistry: 35, 75–139.

Hu BL, et al. 2011. New anaerobic, ammonium-oxidizing community enriched from peat soil. Applied and Environmental Microbiology: 77, 966 –971.

Huang, S., Chen, C., Yang, X., Wu, Q., Zhang, R. 2011. Distribution of typical denitrifying functional genes and diversity of the nirS-encoding bacterial community related to environmental characteristics of river sediments. Biogeosciences Discussions: 8, 5251–5280.

Hubbard, Jr. K.A., Lautz, L.K., Mitchell, M.J., Mayer, B., Hotchkiss, ER. 2010 Evaluating nitrate uptake in a Rocky Mountain stream using labelled ^{15}N and ambient nitrate chemistry. Hydrological Processes: 24, 3322–3336.

Hughes, J.E., Deegan, L.A., Peterson, B.J., Holmes, R.M., Fry, B. 2000. Nitrogen flow through the food web in the oligohaline zone of a New England Estuary. Ecology 81:433-452.

Hulot, F.D., Lacroix, G., Loreau, M. 2014. Differential responses of size-based functional groups to bottom–up and top–down perturbations in pelagic food webs: a meta-analysis. Oikos: 123, 1291–1300

Hunter, M.D., Price, P. 1992. Playing chutes and ladders: Heterogeneity and the relative roles of bottom-up and topdown forces in natural communities. Ecology: 73,724-732.

Humbert, S., Zopfi, J., Tarnawski, S.E. 2012. Abundance of anammox bacteria in different wetland soils. Environmental Microbiology Reports: 4(5), 484–490.

Inger, R. and Bearhop, S. 2008. Applications of stable isotope analyses to avian ecology. Ibis: 150, 447–461.

Inglett, P.W. and Reddy, K.R. 2006. Investigating the use of macrophyte stable C and N isotope ratios as indicators of wetland eutrophication: Patterns in the P-affected Everglades. Limnology and Oceanography: 51, 2380–2387.

Ings, T.C., Montoya, J.M., Bascompte, J., Blüthgen, N., Brown, L., Dormann, C.F., Edwards, F., Figueroa, D., Jacob, U., Jones, J.I., Lauridsen, R.B., Ledger, M.E., Lewis, H.M., Olesen, J.M., Van Veen, F.J., Warren, P.H., Woodward, G. 2009. Ecological networks – beyond food webs. Journal of Animal Ecology: 78, 253–269.

Ives, A.R., Dennis, B., Cottingham, K.L., Carpenter, S.R. 2003. Estimating community stability and ecological interactions from time-series data. Ecological Monographs: 73, 301–330.

Jackson, M.C., Donohue, I., Jackson, A.L., Britton, J.R., Harper, D.M., Grey, J. 2012. Population-level metrics of trophic structure based on stable isotopes and their application to invasion ecology: PLos One, 2, e31757.

Jackson, D.A., Peres-Neto, P.R. and Olden J.D. 2001. What controls who is where in freshwater fish communities — the roles of biotic, abiotic, and spatial factors. Canadian Journal of Fisheries and Aquatic Sciences: 58, 157–170.

Jackson, A.L., Inger, R., Parnell, A.C., Bearhop, S. 2011. Comparing isotopic niche widths among and within communities: SIBER – Stable Isotope Bayesian Ellipses in R. Journal of Animal Ecology: 80, 595–602.

Jansson, M., Andersson, R., Berggren, H., Leonardson, L. 1994. Wetlands and lakes as nitrogen traps. Ambio: 23, 320–325.

Jäntti, H., Stange, C.F. Leskinen, E., Hietanen S. 2011. Seasonal variation in nitrification and nitrate-reduction pathways in coastal sediments in the Gulf of Finland, Baltic Sea. Aquatic Microbial Ecology: 63, 171–181.

Jäntti, H. and Hietanen, S. 2012. The Effects of Hypoxia on Sediment Nitrogen Cycling in the Baltic Sea. AMBIO: 41(2), 161-169.

Jensen, J. P., Kristensen, P., Jeppesen, E. 1990. Relationships between nitrogen loading and in-lake nitrogen concentrations in shallow Danish lakes. Verhandlungen des Internationalen Verein Limnologie: 24, 201–204.

Jeppesen, E., Meerhoff, M., Holmgren, K., Gonzalez-Bergonzoni I., Teixeira-de Mello F., Declerck S.A.J., De Meester L., Sondergaard M., Lauridsen T.L., et al. 2010. Impacts of climate warming on lake fish community structure and potential ecosystem effects. Hydrobiologia: 646, 73-90.

Jeppesen, E., Sondergaard, M., Lauridsen, T.L., Liboriussen, L., Bjerring, R., Johansson, L.S., Landkildehus, F., Kronvang, B., Andersen, H.E., Trolle, D., Jorgensen, T. 2012b. Recent climate induced changes in freshwaters in Denmark. In Climatic Changes and Global Warming of Inland Waters: Impacts ad Mitigation for Ecosystem and Societies, Charles, R.G., Kumagai, M., Robarts, R.D (eds). John Wiley and Sons, Ltd.

Jetten, M.S., Van Niftrik, L., Strous, M., Kartal, B., Keltjens, J.T., Op den Camp, H.J. 2009. Biochemistry and molecular biology of anammox bacteria. Critical Reviews in Biochemistry and Molecular Biology: 44, 65–84.

Jones, J.I. and Waldron, S. 2003. Combined stable isotope and gut contents analysis of food webs in plant-dominated, shallow lakes. Freshwater Biology: 48, 1396–1407.

Jones, R.I., King, L., Dent, M.M., Marberly, S.C., Gibson, C.E. 2004. Nitrogen stable isotope ratios in surface sediments, epilithon and macrophytes

from upland lakes with differing nutrient status. Freshwater biology: 49(4), 382-391.

Johnson, L.T., Royer, T.V., Edgerton, J.M. Leff, L.G. 2012. Manipulation of the dissolved organic carbon pool in an agricultural stream: responses in microbial community structure, denitrification, and assimilatory nitrogen uptake. Ecosystems:15, 1027–1038.

Jopp, F., Reuter, H., Breckling, B., (eds). 2011. Modelling complex ecological dynamics: an Introduction into ecological modelling for students, Teachers and Scientists. Berlin: Springer-Verlag.

Karr, J.R. 1981. Assessment of biotic integrity using fish communities. Fisheries: 6(6), 21-27.

Kartal, B., Kuypers, M., Lavik, G., Schalk, J., Op den Camp H., Jetten, M., Strous, M. 2007. Anammox bacteria disguised as denitrifiers: nitrate reduction to dinitrogen gas via nitrite and ammonium. Environmental Microbiology: 9, 635–642.

Kazanci, C.K. 2007. EcoNet, A new software for Ecological Model Simulation and Network Analysis. Ecological Modelling: 208, 3-8.

Keller, D.R., and Golley, F.B. , (eds). 2000. The Philosophy of Ecology: From Science to Synthesis. Athens: University of Georgia Press.

Kelly, L.J., Martínez, del Rio C. 2010. The fate of carbon in growing fish: an experimental study of isotopic routing. Physiological and Biochemical Zoology: 83, 473-480.

Kendall, C. 1998. Tracing nitrogen sources and cycles in catchments. In: Kendall, C. and McDonnell, J.J. (eds). Isotope tracers in catchment hydrology. Elsevier Science, Amsterdam.

Kendall, C. and McDonnell, J.J. (eds). 1998. Isotope Tracers in Catchment Hydrology. Elsevier Science: Amsterdam, The Netherland.

Kendall, C. and Doctor, D.H. 2003. Stable Isotope Applications in Hydrologic Studies, In Treatise on Geochemistry, Oxford.

Kendall, C., Young, M.B., Silva, S.R. 2010. Applications of stable isotopes for regional to national-scale water quality monitoring programs. In: West JB, Bowen GJ, Dawson TE, Tu KP (eds) Isoscapes: understanding movement, pattern, and process on Earth through isotope mapping. Springer, Dordrecht.

Keough, J.R., Sierszen, M.E., Hagley, C.A. 1996. Analysis of a Lake Superior Coastal Food Web with Stable Isotope Techniques: Limnology and Oceanography: 41(1),136-146.

Kemp, M.J., and Dodds, W.K. 2002. Comparisons of nitrification and denitrification in prairie and agriculturally influenced streams. Ecological Applications: 12, 998–1009.

Kim, O.S., Imhoff, J.F., Witzel, K.P., Junier, P. 2011. Distribution of denitrifying bacterial communities in the stratified water column and sediment–water interface in two freshwater lakes and the Baltic Sea. Aquatic Ecology: 45, 99–112.

Kling, G.W. 1994. Ecosystem scale experiments: the use of stable isotopes in freshwaters. In: Baker LA (ed) Environmental chemistry of lakes and reservoirs. American Chemical Society, Washington DC.

Kling, G.W., Kipphut, G.W., Miller, M.C., O'Brien, W.J. 2000. Integration of lakes and streams in a landscape perspective: the importance of material processing on spatial patterns and temporal coherence. Freshwater Biology: 43, 477–497.

Kowalchuk, G.A., Stephen, J.R. 2001. Ammonia-oxidizing bacteria: A model for molecular microbial ecology. Annual Review of Microbiology: 55, 485–529.

Kritzberg, E.S., Cole, J.J., Pace, M.L., Graneli, W., Bade, D.L. 2004. Autochthonous versus allochthonous carbon sources of bacteria: Results from whole-lake ^{13}C addition experiments. Limnology and Oceanography: 49, 588-596.

Kritzberg, E.S., Cole, J.J., Pace, M.L., Graneli, W. 2006. Bacterial growth on allochthonous carbon in humic and nutrient-enriched lakes: results from whole-lake ^{13}C addition experiments. Ecosystems: 9,489-499.

Kulkarni, M.V., Groffman, P.M., Yavitt, J.B. 2008. Solving the global nitrogen problem: It is a gas! Frontiers in Ecology and the Environment: 6,199–206.

Kuypers, M., Sliekers, A., Lavik, G., Schmid, M., Kuenen, J., Damste, J., Strous§, M., Jetten, M. 2003. Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. Letters to nature: 422, 608-611.

Kuypers, M., Lavik, G., Woejen, D., Schmid, M., Fuhs, B., Amann, R., Jørgensen, B., Jetten, M. 2005. Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. Proceedings

of the National Academy of Sciences of the United State of America: 102, 6478–6483.

Lampert, W. and Sommer, U. 1997. Limnoecology. Oxford University Press.

Layman, C.A., Arrington, D.A., Montaña, C.G., Post, D.M. 2007. Can stable isotope ratios provide for community-wide measures of trophic structure? Ecology: 88, 42–48.

Layman, C.A. and Post, D.M. 2008. Can stable isotope ratios provide for community-wide measures of trophic structure ? Reply. Ecology: 89(8), 2358-2359.

Layman, C.A., Araujo, M.S., Boucek, R., Hammerschlag-Peyer, C.M., Harrison, E., Jud, Z.R., Matich, P., Rosenblatt, A.E., Vaudo, J.J., Yeager, L.A., and others. 2012. Applying stable isotopes to examine food-web structure: An overview of analytical tools. Biological Reviews: 87, 454–562.

Larsen, D.R. and Speckman. 2004. Multivariate Regression Trees for Analysis of Abundance Data. Biometrics: 60(2), 543-549.

Lawton, J.H. 1989. Food webs. In: Cherrett, J.M. (ed) Ecological concepts: the contribution of ecology to an understanding of the natural world. Blackwell, Oxford.

Lee, K.M., Lee, S.Y., Connolly, R.M. 2011. Combining stable isotope enrichment, compartmental modelling and ecological network analysis for quantitative measurement of food web dynamics. Methods in Ecology and Evolution: 2, 56-65.

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., Prosser, J.I., Schuster, S.C., Schleper, C. 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature: 442, 806– 809.

Lehmann, M.F., Sigman, D.M., Berelson, W.M. 2004. Coupling the N-15/N-14 and O-18/O-16 of nitrate as a constraint on benthic nitrogen cycling, Marine Chemistry: 88, 1–20.

Li, K., Liu, Z., Gu, B. 2010. The fate of cyanobacterial blooms in vegetated and unvegetated sediments of a shallow eutrophic lake: A stable isotope tracer study. Water Research: 4, 1591–1597.

Likens, G.E. 1992. The ecosystem approach: its use and abuse. Ecology Institute, Oldendorf/Luhe, Germany.

Limpiyakorn, T., Sonthiphand, P., Rongsayamanont, C., Polprasert, C. 2011. Abundance of amoA genes of ammonia-oxidizing archaea and bacteria in

activated sludge of full-scale wastewater treatment plants. Bioresource Technology: 102, 3694–3701.

Lin, A.Y-C., Debroux ,J.F., Cunningham, J.A., Reinhard, M. 2003. Comparison of rhodamine WT and bromide in the determination of hydraulic characteristics of constructed wetlands. Ecological Engineer: 20, 75-88.

Lindeman, R., L. 1942. The trophic-dynamics aspect of ecology. Ecology: 23, 399-418.

Lis, G., Wassenaar, LI., Hendry, M.J. 2008. High-precision laser spectroscopy D/H and $^{18}O/^{16}O$ measurements of microliter natural water samples. Analytical Chemistry: 80, 287-293.

López-Gutiérrez, J.C., Henry, S., Hallet, S. Martin-Laurent, F., Catroux, G., Philippot, L. 2004. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR, Journal of Microbiological Methods: 57(3), 399-407.

Mantyka-Pringle, C.S., Martin, T.G., Moffatt, D.B., Linke, S., Rhodes, J.R., 2014. Understanding and Predicting the Combined Effects of Climate Change and Land-Use Change on Freshwater Macroinvertebrates and Fish. Journal of Applied Ecology: 5 (3), 572-581.

Margalef, R. 1968. Perspectives in ecological theory. University of Chicago Press. 111 pp.

Mariotti, A. 1984. Atmospheric nitrogen is a reliable standard for natural l SN abundance measurements. Nature 311:251-252.

Martinez del Rio, C., Wolf, N., Carleton, S.A., Gannes, L.Z. 2009. Isotopic ecology ten years after a call for more laboratory experiments. Biological Reviews: 84, 91-111.

May, R.M. 1983. The structure of food webs. Nature: 301, 566-568.

Marker, A.F.H., Nusch, E.A., Rai, H. and Rieman, B. 1980. The measurement of photosynthetic pigments in freshwater and standarisation of methods: conclusions and recommendations. Archiv für Hydrobiologie, Ergebnisse der Limnologie 14: 91–106.

Martí, E., and Sabater, F. 1996. High variability in temporal and spatial nutrient retention in Mediterranean streams. Ecology, 77: 854–869.

Mbabazi, D., Makanga, B., Orach-Meza, F., Hecky, R.E., Balirwa, J.S., Ogutu-Ohwayo, R., Verburg, P., Chapman, L., Muhumuza, E. 2009. Intra-lake stable isotope ratio variation in selected fish species and their possible

carbon sources in Lake Kyoga (Uganda): implications for aquatic food web studies. African Journal of Ecology: 48, 667–675.

McCann, K.S. 2000. The diversity-stability debate. Nature: 405, 228-233.

Mcnaughton, S.J. 1978. Stability and diversity in ecological communities. Nature: 274,251-252.

Macfadyen, S., Gibson, R., Polaszek, A., Morris, R.J., Craze, P.G., Planque, R., Symondson, W.O.C., Memmott, J. 2009. Do differences in food web structure between organic and conventional farms affect the ecosystem service of pest control? Ecology Letters: 12, 229–238.

McIntosh, R.P. 1985. The background of ecology. Cambridge University Press, Cambridge, UK.

Melián, C.J. and Bascompte J. 2004. Food web cohesion. Ecology : 85 (2), 352-358.

Menzel, D. W. and Corwin, N. 1965. The measurement of total phosphorus in seawater based on the liberation of organically bound fractions by persulfate oxidation. Limnology and Oceanography: 10, 280–282.

Merriam, J.L., McDowell, W.H., Tank, J.L., Wollheim, W.M., Crenshaw, C.C., Johnson, S.L. 2002. Nitrogen dynamics, retention and transport in a tropical stream. Freshwater Biology: 47, 143-160.

Meyer, J.L., Paul, M.J, Taulbee, W.K. 2005. Stream ecosystem function in urbanizing landscapes. Journal of the North American Benthological Society: 24, 602–612.

Middleburg, J.J., Barranguet, C., Boschker, H.T., Herman, M.J., Moens, T., Heip, C.H .2000. The fate of intertidal microphytobenthos carbon: an in situ ^{13}C-labeling study. Limnology and Oceanography: 45,1224-1234.

Middelburg, J.J. and Nieuwenhuize, J. 2001. Nitrogen isotope tracing of dissolved inorganic nitrogen behaviour in tidal estuaries. Estuarine, Coastal and Shelf Science: 53, 385–391.

Middelburg, J.J. 2014. Stable isotopes dissect aquatic food webs from the top to the bottom. Biogeosciences: 11, 2357-2371.

Michener, R. and Lajtha, K. (eds). 2007. Stable Isotopes in Ecology and Environmental Science. Oxford, UK: Blackwell.

Minagawa, M. and Wada, E. 1984. Stepwise enrichment of ^{15}N along food chains: further evidence and the relation between d^{15}N and animal age. Geochimica et Cosmochimica Acta 48: 1135–1140.

Mincer, T.J., Church, M.J., Taylor, L.T., Preston, C., Karl, D.M., DeLong, E.F. 2007. Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North Pacific Subtropical Gyre. Environmental Microbiology: 9, 1162–1175.

Meyer, R.L., Risgaard-Petersen, N., Allen, D.E. 2005. Correlation between Anammox Activity and Microscale Distribution of Nitrite in a Subtropical Mangrove Sediment. Applied and Environmental Microbiology: 71(10), 6142-6149.

Moffat, A.S. 1998. Global nitrogen overload problem grows critical. Science: 279, 988–989.

Mohan, S.B., Schmid, M., Jetten, M., Cole, J. 2004. Detection and widespread distribution of the nrfA gene encoding nitrite reduction to ammonia, a short circuit in the biological nitrogen cycle that competes with denitrification. FEMS Microbiology Ecology: 49, 433–443.

Moore, J.C. and Hunt, H.W. 1988. Resource compartmentation and the stability of real ecosystems. Nature: 314, 261-263.

Moore, J.W. and Semmens, B.X. 2008. Incorporating uncertainty and prior information into stable isotope mixing models. Ecology Letters: 11, 470–480.

Moreno, C.E, Arita, H.T., Solis, L. 2006. Morphological assembly mechanisms in Neotropical bat assemblages ad ensembles within a landscape. Oecologia: 149, 133-140.

Morrall, D.D., Christman, S.C., Peterson, B.J., Wolheim, W.M., Belanger, S.E. 2006. Utility of stable isotope (C^{13} and N^{15}) to demonstrate comparability between natural and experimental stream for environmental risk assessments. Ecotoxicology and Environmental Safety: 65, 22-35

Mulholland, P.J., Tank J.L., Sanzone, D.M., Wollheim, W., Peterson, B.J., Webster, J.R., Meyer, J.L. 2000a. Nitrogen cycling in a deciduous forest stream determined from a tracer ^{15}N addition experiment in Walker Branch, Tennessee. Ecological Monographs: 70, 471-493.

Mulholland, P.J., Tank, J.L., Sanzone, D.W., Wolheim, W.M., Peterson, B.J., Webster, J.R., Meyer, J.L. 2000b. Food resources of stream macroinvertebrates determined by natural-abundance stable C and N isotopes and a ^{15}N tracer addition. Journal of the North American Benthological Society: 19, 145-157.

Mulholland, P.J., Tank, J.L., Sanzone, D.M., Webster, J.R., Wollheim, W., Peterson, B.J., Meyer, J.L. 2001. Ammonium and nitrate uptake lengths in a small forested stream determined by ^{15}N tracer and short-term nutrient enrichment experiments. Verhandlungen des Internationalen Verein Limnologie: 27:1320-1325.

Mulholland, P.J., Fellows, C.S., Tank, J.L., Grimm, N.B., Webster, J.R., Hamilton, S.K., Marti, E., Ashkenas, L., Bowden, W.B., Dodds, W.K., and others. 2001. Inter-biome comparison of factors controlling stream metabolism. Freshwater Biology: 46, 1503-1517.

Mulholland, P.J., Valett, H.M., Webster, J.R., Thomas, S.A., Cooper, L.W., Hamilton, S.K., Peterson, B.J. 2004. Stream denitrification and total nitrate uptake rates measured using a field ^{15}N tracer addition approach. Limnology and Oceanography: 49, 809-820.

Mulholland, P.J., Helton, A.M., Poole, G.C., Hall, Jr. R.O., Hamilton, S.K., Peterson, B.J., Tank, J.L., Ashkenas, L.R., Cooper, L.W., Dahm, C.N., and others. 2008. Stream denitrification across biomes and its response to anthropogenic nitrate loading. Nature: 452, 202-205.

Mulholland, P.J., Hall, Jr. R.O., Sobota, D.J., Dodds, W.K., Findlay, S., Grimm, N.B., Hamilton, S.K., McDowell, W.H., O'Brien, J.M., Tank, J.L., and others. 2009. Nitrate removal in stream ecosystems measured by ^{15}N addition experiments: denitrification. Limnology and Oceanography 54: 666-680

Mutshinda, C.M, and O'Hara, R.B. 2011. Integrating the niche and neutral perspectives on community structure and dynamics. Oecologia: 166, 241–251.

Neubauer, S.C., Andersson, I.C., Neikirk, B.B. 2005. Nitrogen cycling and ecosystem exchange in a Virginia tidal freshwater marsh. Estuaries: 28, 909–922.

Newbold, J.D. Elwood, J.W., O´Neill, R.V., Van Winkle, W. 1981. Measurig nutrient spiraling in streams. Canadian Journal of Fisheries and Aquatic Sciences: 38, 860-863.

Newbold, J.D. O´Neill, R.V., Elwood, J.W., Van Winkle, W. 1982. Nutrient spiralling in streams: implications for nutrient limitation and invertebrates activity. American Naturalist: 120, 628-652.

Newsome, S.D., del Rio, C.M., Bearhop, S., Phillips, D.L. 2007. A niche for isotopic ecology. Frontiers in Ecology and the Environment: 5, 429–436.

Nogales, B., Timmis, K.N., Nedwell, D.B., Osborn, A.M. 2002. Detection and Diversity of Expressed Denitrification Genes in Estuarine Sediments after Reverse Transcription-PCR Amplification from mRNA. Applied and Environmental Microbiology: 68(10), 5017.

O'Brien, J.M., Dodds, W.K., Wilson, K.C., Murdoch, J.N., Eichmiller, J. 2007. The saturation of N cycling in Central Plains streams: ^{15}N experiments across a broad gradient of nitrate concentrations. Biogeochemistry: 84, 31–49.

O'Brien, J.M., Hamilton, S.K., Podzikowski, L., Ostrom, A. 2012. The fate of assimilated nitrogen in streams: an in situ benthic chamber study. Freshwater Biology: 57, 1113–1125.

Olsson, K., Stenroth, P., Nyström, P., Granéli, W. 2009. Invasions and niche width: does niche width of an introduced crayfish differ from a native crayfish? Freshwater Biology: 54, 1731–1740.

O'Leary, M.H. 1988. Carbon Isotopes in Photosynthesis using stable isotopes. Bioscience: 38, 328–336.

Ohte, N., Tayasu, I., Kohzu, A., Yoshimizu, C., Osaka, K., Makabe, A., Koba, K., Yoshida, N., Nagata, T. 2010. Spatial distribution of nitrate sources of rivers in the Lake Biwa watershed, Japan: controlling factors revealed by nitrogen and oxygen isotope values. Water Resources Research: 46, 7.

Orghidan, T. 1959. Ein neuer lebensraum des unterirdischen wassres der hyporheischen biotope. Archiv für Hydrobiologie: 55, 392-414.

Pace, M.L., Cole, J.J., Carpenter, S.R., Kitchell, J.F., Hodgson, J.R., Van de Bogert, M.C., Bade, D.L., Kritzberg, E.S., Bastviken, D. 2004. Whole-lake carbon-^{13} additions reveal terrestrial support of aquatic food webs. Nature: 427, 240-243.

Pace, M.L., Carpenter, S.R., Cole, J.J., Coloso, J.J., Kitchell, J.F., Hodgson, J.R., Middleburg, J.J., Preston, N.D., Solomon, C.T., Weidel, B.C. 2007. Does terrestrial organic carbon subsidize the planktonic food web in a clear-water lake. Limnology and Oceanography: 52, 2177-2189.

Page, K. L., Dalal, R. C., Menzies, N. W. 2003. Nitrate ammonification and its relationship to the accumulation of ammonium in a vertisol subsoil. Australian Journal of Soil Research: 41, 687–697.

Pastor, A. Nitrogen Sable Isotopes in primary uptake compartments across streams differing in nutrient availability. 2013. Environmental Science and Technology: 47, 10155−10162.

Parkyn, S.M., Quinn, J.M., Cox, T.J., Broekhuizen, N. 2005. Pathways of N and C uptake and transfer in stream food webs: an isotope enrichment experiment. Journal of the North American Benthology Societ: 24, 955-975.

Parnell, A., Inger, R., Bearhop, S., Jackson, A.L. 2008. SIAR: Stable isotope analysis in R. Available at: http://cran.r-project.org/web/packages/ siar.

Parnell, A.C., Inger, R., Bearhop, S. and Jackson, A.L. 2010. Source partitioning using stable isotopes: Coping with too much variation. PLoS One: 5, e9672.

Pauer, J.J. and Auer, M.T. 2000. Nitrification in the water column and sediment of a hypereutrophic lake and adjoining rover system. Water Research: 34(4), 1247-1254.

Penton, C.R., Devol, A.H., Tiedje, J.M. 2006. Molecular evidence for the broad distribution of anaerobic ammonium-oxidizing bacteria in freshwater and marine sediments. Applied Environmental Microbiology: 72(10), 6829–6832.

Petchey, O.L., McPhearson, P.T., Casey, T.M., Morin, P.J. 1999. Environmental warming alters food-web structure and ecosystem function. Nature: 40(2), 69-72.

Peterson, B.J., Howarth, R.W., Garritt, R.H. 1985. Multiple stable isotopes used to trace organic matter in estuarine food webs. Science: 227, 1361–1363.

Peterson, B.J., Fry, B., 1987. Stable isotopes in ecosystem studies. Annual Review Ecology, Evolution and Systematics: 18, 293–320.

Peterson, B.J., Bahr, M., Kling, G.W. 1997. A tracer investigation of nitrogen cycling in a pristine tundra river. Canadian Journal of Fisheries and Aquatic Science: 54, 2361–2367.

Peterson, B.J. 1999. Stable isotopes as tracers of organic matter input and transfer in benthic food webs: a review. Acta Oecologia: 20, 479–487.

Peterson, B.J., Wollheim, W.M., Mulholland, P.J., Webster, J.R., Meyer, J.L., Tank, J.L., Marti, E., Bowden, W.B., Valett, H.M., Hershey, A.E., and others. 2001. Control of nitrogen export from watersheds by headwater streams. Science: 292(5514): 86–90.

Peterson, B., Tobias, C., Van Drecht, G. 2006. Denitrification across landscapes and waterscapes: a synthesis. Ecological Applications: 16, 2064–2090.

Perkins, D.M., McKie, B.G., Malmqvist, B., Gilmour, S.G., Reiss, J., Woodward, G. 2010. Environmental warming and biodiversity

ecosystem functioning in freshwater microcosms: partitioning the effects of species identity, richness and metabolism. Advances in Ecological Research: 43, 177-209.

Perkins, M.J. 2013. Quantifying the effects of biodiversity on food web structure: a stable isotope approach. Dissertation. University of Exeter.

Perkins, M.J., McDonald, R.A., Van Veen, F.J.F., Kelly, S.D., Rees, G., Bearhop, S. 2013. Important impacts of tissue selection and lipid extraction on ecological parameters derived from stable isotope ratios. Methods in Ecology and Evolution: 4, 944-953.

Perryman, S.E., Rees, G.N., Walsh, C.J. 2008. Analysis of denitrifying communities in streams from an urban and non-urban catchment. Aquatic Ecology: 42, 95–101.

Phillips, D.L. 2001. Mixing models in analyses of diet using multiple stable isotopes: a critique. Oecologia: 127, 166–170.

Phillips, D.L and Gregg, J.W. 2001. Uncertainty in source partitioning using stable isotopes. Oecologia: 127, 171–179.

Phillips, D.L. and Gregg, J.W. 2003. Source partitioning using stable isotopes: coping with too many sources. Oecologia 136:261-269.

Phillips, D.L. and Koch, P.L. 2002. Incorporating concentration dependence in stable isotope mixing models. Oecologia: 130, 114–125.

Philippot, L. and Hallin, S. 2005. Finding the missing link between diversity and activity using denitrifying bacteria as a model functional community. Microbiology: 8(3), 234-239.

Piatek, K.B., Mitchell, M.J., Silva, S.R., Kendall, C. 2005. Sources of nitrate in snowmelt discharge: Evidence from water chemistry and stable isotopes of nitrate. Water, Air and Soil Pollution: 165, 13–36.

Piña-Ochoa, E. and Álvarez-Cobelas, M. 2006. Denitrification in aquatic environments: a cross-system analysis. Biogeochemistry: 81, 111–130.

Pimm, S.L. 1982. Food Webs. Chapman and Hall, London.

Pimm, S.L., 1984. The complexity and stability of ecosystems. Nature: 307, 321-326.

Pimm, S.L. 2002. Food Webs. University of Chicago Press, Chicago.

Pinnegar, J.K., Polunin, N.V.C. 1999. Differential fractionation of $\delta^{13}C$ and $\delta^{15}N$ among fish tissues: implications for the study of trophic interactions. Functional Ecology: 13, 225-231.

Post, D.M., Pace, M.L., Hairnton, N.G. 2000. Ecosystem size determinates food-chain length in lakes. Nature: 405, 1047-1049.

Post, D.M. 2002. Using stable isotopes to estimate trophic position: models, methods, and assumptions. Ecology: 83, 703–718.

R Development Core Team. 2007. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.

Raikow, D.F. and Hamilton, S.K. 2001. Bivalve diets in a midwestern U.S stream: A stable isotope enrichment study. 2001. Limnology and Oceanography: 46, 514-522.

Redfield, A.C., Ketchum, B.H., and Richards F.A. 1963. The influence of organisms on the composition of sea water. In The Sea Vol. 2, Hill, M. N. (eds). Wiley-Interscience, New York.

Riera, P., Stal, L.J., Nieuwenhuize, J., Richard, P., Blanchard, G, Gentil, F. 1999. Determination of food sources for benthic invertebrates in a salt marsh (Aiguillon Bay, France) by carbon and nitrogen stable isotopes: importance of locally produced sources. Marine Ecology Progress Series: 187, 301–307.

Rissanen, Antti. 2012. Nitrogen removal by microbial processes in aquatic systems Jyväskylä. Thesis dissertation University of Jyväskylä. Jyväskylä. Studies in Biological and Environmental Science.

Risgaard-Petersen, N. 2003. Coupled nitrification-denitrification in autotrophic and heterotrophic estuarine sediments: on the influence of benthic microalgae. Limnology and Oceanography: 48, 93–105.

Risgaard-Petersen N., Nicolaisen, M.H., Revsbech, N.P., Lomstein, B.A. 2004. Bacteria and Benthic Microalgae Competition between Ammonia-Oxidizing. Applied and Environmental Microbiology: 70(9), 5528.

Riss, T., Dodds, W.K., Kristensen, P.B., Bairner, A.J., 2012. Nitrogen cycling and dynamics in a macrophyte-rich stream as determinated by $^{15}N\text{-}NH_4$ release Freshwater Biology: 57, 1579-1591.

Ricklefs, R.E. and Travis, J. 1980. A morphological approach to the study of avian community organization. Auk: 97, 321–338.

Ricklefs, R.E. and Miles, D.B. 1994. Ecological and evolutionary inferences from morphology: an ecological perspective. In ecological morphology: integrative organismal biology, Wainwright and S.M. Reilly (eds). University of Chicago Press, Chicago.

Ricklefs, R.E. 2012. Species richness and morphological diversity of passerine birds. Proceedings of the National Academy of Sciences: 109(36), 14482-14487.

Rockstrom, J., Steffen, W., Noone, K., Persson, A., Chapin III, F.S., Lambin, E.F., Lenton, T., Scheffer, M., Folke, C., Schellnhuber, H.J., and others. 2009. A safe operating space for humanity. Nature: 461, 472– 475.

Ryding, S.O. and Rast W. 1992. El control de la eutrofización en lagos y pantanos. UNESCO. Ed. Pirámide, S.A. Madrid.

Rotthauwe, J. H., Witzel, K.P., Liesack, W. 1997. The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Applied and Environmental Microbiology: 63(12), 4704-4712.

Rütting, T., Boeckx, P., C. Müller, C., Klemedtsson, L. 2011. Assessment of the importance of dissimilatory nitrate reduction to ammonium for the terrestrial nitrogen cycle. Biogeosciences: 8, 1779–1791.

Rysgaard, S., Risgaard-Peterson, N., Nielsen, L.P., Revsbech, N.P. 1993. Nitrification and denitrification in lake and estuarine sediments measured by the ^{15}N dilution technique and isotope pairing. Applied and Environmental Microbiology: 59, 2093–2098.

Robinson, D. 2001. $\delta^{15}N$ as an integrator of the nitrogen cycle ends. Ecology and Evolution: 16 (3), 153 - 162.

Sanzone, D. M. 2001. Linking communities across ecosystem boundaries: the influence of aquatic subsidies on terrestrial predators. Dissertation. University of Georgia, Athens, Georgia, USA.

Sanzone, D.M., Meyer, J.L., Marti, E., Gardiner, E.P., Tank, J.L., Grimm, N.B. 2003. Carbon and nitrogen transfer from a desert stream to riparian predators. Oecologia 134:238–250

Sardans, J., Peñuelas, J., Rivas-Ubach, A. 2011. Ecological metabolomics: overview of current developments and future challenges. Chemoecology: 21, 191–225.

Saunders, D.L. and Kalff, J. 2001. Nitrogen retention in wetland, lakes and rivers. Hydrobiologia: 443, 205–212.

Scott, J.T., McCarthy, M.J., Gardner, W.S., Doyle, R.D. 2008. Denitrification, dissimilatory nitrate reduction to ammonium, and nitrogen fixation along a nitrate concentration gradient in a created freshwater wetland. Biogeochemistry: 87, 99–111.

Schaefer, S.C. and Alber, M. 2007. Temperature controls a latitudinal gradient in the proportion of watershed nitrogen exported to coastal ecosystems. Biogeochemistry: 85(3), 333–346.

Scheffer, M. 1998. Ecology of shallow lakes. Chapman and Hall, London.

Scheffer, M. 1998. Ecology of shallow lakes. Population and Community Biology Series, Kluwer Academic Publisher. Dordrecht.

Schindler, D.E., Carpenter, S.R., Cole, J.J., Kitchell, J.F., Pace, M.L. 1997. Influence of food web structure on carbon exchange between lakes and the atmosphere. Science: 277, 248–251.

Schimel, D.S. (ed). 1993. Theory and application of tracers. Academic Press, San Diego

Schmid, M.C., Risgaard-Petersen, N., Van de Vossenberg, J., Kuypers, M.M., Lavik, G., Petersen, J., Hulth, S., Thamdrup, B., Canfield, D., Dalsgaard, T., and others. 2007. Anaerobic ammonium-oxidizing bacteria in marine environments: Widespread occurrence but low diversity. Environmental Microbiology: 9 (6), 1476–1484.

Schmidt, S.N., Olden, J.D., Solomon, C.T., Vander Zanden, M.J. 2007. Quantitative approaches to the analysis of stable isotope food web data. Ecology: 88 (11), 793-802.

Schubert, C.J., Durisch-Kaiser, E., Wehrli, B., Thamdrup, B., Lam, P., Kuypers, M. 2006. Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika). Environmental Microbiology: 8, 1857–1863.

Shuttleworth, W. J. 1993 Evaporation. In Maidment, D. R. (Ed.) Handbook of Hydrology. New York, McGraw-Hill Inc.

Seitzinger, S.P. 1988. Denitrification in freshwater and coastal marine systems: ecological and geochemical significance. Limnology and Ocenaography: 33, 702,724.

Seitzinger, S., Harrison, J.A., Böhlke, J.K., Bouwman, A.F., Lowrance, R., Peterson, B., Tobias, C., Van Drecht, G. 2006. Denitrification across landscapes and waterscapes: a synthesis. Ecological Applications: 16, 2064–2090.

Semmens, B.X., Ward, E.J., Moore, J.W., Darimont, C.T. 2009. Quantifying inter- and intrapopulation niche variability using hierarchical Bayesian stable isotope mixing models. PLoS ONE 4:e6187.

Shannon, C. E. and Weaver, W. 1948. A mathematical theory of communication. The Bell System Technical Journal: 27, 379–423 and 623–656.

Shurin, J.B., Clasen, J.L., Greig, H.S., Kratina, P., Thompson, P.L., 2012. Warming shifts top-down and bottom-up control of pond food web structure and function. Philosophical Transactions B: 367, 3008–3017.

Sigman, D.M., Altabet, M.A., Michener, R., McCorkle, C.D., Fry, B., Holmes, R.M. 1997. Natural abundance-level measurement of the nitrogen isotopic composition of oceanic nitrate: An adaptation of the ammonia diffusion method. Marine Chemistry: 57, 227–242.

Simon, K.S., Benfield, E.F., Macko, S.A. 2003. Food web structure and the role of epilithic biofilms in cave streams. Ecology: 84, 2395-2406.

Smith, C.J., Nedwell, D.B., Dong, L.F., Osborn, A.M. 2007. Diversity and abundance of nitrate reductase genes (narG and napA), nitrite reductase genes (nirS and nrfA), and their transcripts in estuarine sediments. Applied and Environmental Microbiology: 73, 3612–3622.

Smith, C.J. and Osborn, A.M. 2009. Minireview. Advantages and limitations of quantitative PCR(Q-PCR)-based approaches in microbial ecology. FEMS Microbiology Ecology: 67, 6–20.

Smith, F. 1969. Effects of enrichment in mathematical models. In Eutrohication: causes, consequences and correctives. National Academy of Siences.

Solomon, C.T., Carpenter, S.R., Cole, J.J., Pace, M.L. 2008. Support of benthic invertebrates by detrital resources and current autochthonous primary production: results from a whole-lake ^{13}C addition. Freshwater Biology: 53, 42–54.

Solomon, C.T., Carpenter, S.R., Clayton, M.K., Cole, J.J., Coloso, J.J., Pace, M.L., Vander Zander, M.J., Weidel, B.C. 2011. Terrestrial, benthic, and pelagic resource use in lakes: results from a three-isotope Bayesian mixing model. Ecology: 92, 1115–1125.

Solorzano, L. 1969. Determination of ammonia in natural waters by the phenol-hypochlorite method. Limnology and Oceanography: 14, 799–801.

Sorensen, P., and Jensen, E.S.. 1991. Sequential diffusion of ammonium and nitrate from soil extracts to a polytetrafluoroethylene trap for ^{15}N determination. Analytica Chimica Acta: 252, 201–203.

Soto, D.X., Wassenaar, L.I., Hobson, K.A. 2013. Stable hydrogen and oxigen isotopes in aquatic food webs are tracers of diet and provenance. Functional Ecology: 27, 535-543.

Staehr, P.A., Bade, D., Van de Bogert, M.C., Koch, G.R., Williamson, C., Hanson, P., Cole, J.J., Kratz, T. 2010. Lake metabolism and the diel oxygen technique: state of the science. Limnology and Oceanography Methods: 8, 628–644.

Stanley, E.S. and Maxted, J.T. 2008. Changes in the dissolves nitrogen pool across land cover gradients in Wisconsin streams. Ecological Applications: 18(7), 1579-1590.

Stephen, A.B., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L. 2009.The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry: 55, 4 611–622.

Stark, J. M. and Hart, S. C. 1997. High rates of nitrification and nitrate turnover in undisturbed coniferous forest. Nature: 385, 61-64.

Sterner, R.W., Elser, J.J., Fee, E.J., Guildford, S.J., Chrzanowski, T.H. 1997. The light: nutrient ratio in lakes: The balance of energy and materials affects ecosystem structure and process. The American Naturalist: 150, 663-684.

Storey, D.R., Williams, D., Fultrhorpe, R.R. 2004. Nitrogen processing in the hiporheic zone of pastoral stream. Biogeochemistry: 69, 285-313.

Strauss, E.A. and Lamberti, G.A. 2000. Regulation of nitrification by organic carbon. Limnology and Oceanography: 45, 1854–1859.

Stream solute workshop. 1990. Concepts and methods for assessing solute dynamics in stream ecosystems. Journal of the North American Benthological Society: 9, 95-l 19.

Strong, D.R. 1988. Food web theory. Ecology: 69, 1647-1676.

Strong, D.R. 1992. Are trophic cascades all wet – differentiation and donor-control in species ecosystems. Ecology: 73, 747–754.

Sulzman, E.W. 2007. Stable isotope chemistry and measurement: a primer. Michener R, Lajtha (eds) Stable isotopes in ecology and environmental science, 2nd edition, Ecological Methods and Concept Series. Blackwell, Malden.

Syväranta, J., Lensu, A., Marjomäki, T.J., Oksanen, S., Jones, R.I. 2013. An empirical evaluation of the utility of convex hull and standard ellipse

areas for assessing population niche widths from stable isotope data. PLoS ONE 8: e56094.

Svendsen, L. M. and Kronvang, B. 1993. Retention of nitrogen and phosphorus in a Danish lowland river system: implications for the export from the watershed. Hydrobiologia: 251, 123–135.

Takimoto, G. and Post, D.M. 2013. Environmental determinants of food-chain length: a meta-analysis. Ecological Research: 28, 675-681.

Therneau, T.M. and Atkinson, B. 2009. Package 'mvpart'.

Thompson, R.M., Brose, U., Dunne, J.A., Hall Jr, R.O., Hladyz, S., Kitching, R.L., Tylianakis, J. M. 2012. Food webs: reconciling the structure and function of biodiversity. Trends in ecology and evolution: 27(12), 689-697.

Taipale, S., Kankaala, P., Tirola, M., Jones, R.I. 2008. Whole-lake dissolved inorganic ^{13}C additions reveal seasonal shifts in zooplankton diet. Ecology: 89, 463-474.

Tank, J.L., Mulholland, P.J., Meyer, J.L., Bowden, J.R., Webster, J.R., Peterson, B.J. 2000a. Contrasting food web linkages for grazing pathway in three temperate forested stream using ^{15}N as a tracer. Verhandlungen des Internationalen Verein Limnologie: 27, 2832-2835.

Tank, J.L., Meyer, J.L., Sanzone, D.W., Mulholland, P.J., Webster, J.R., Peterson, B.J., Wolheim, W.M., Leonard, N.E. 2000b. Analysis of nitrogen cycling in a forest stream during autumn using a ^{15}N tracer addition. Limnology and Oceanography: 45, 1013-1029.

Thompson, R.M., Brose, U., Dunne, J.A., Hall Jr, R.O., Hladyz, S., Kitching, R.L., Tylianakis, J.M. 2012. Food webs: reconciling the structure and function of biodiversity. Trends in ecology and evolution: 27 (12), 689-697.

Throbäck, I.N., Enwall, K., Jarvis, Å., Hallin, S. 2004. Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiology Ecology: 49, 401–417.

Tiedje, J.M., 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In Zehnder, A.J.B. (Ed.), Biology of Anaerobic Microorganisms. Wiley, New York.

Tobias, C. R., Macko, S.A., Anderson, I.C., Canuel, E.A., Harvey. J.W. 2001. Tracking the fate of a high concentration groundwater nitrate plume

through a fringing marsh: A combined groundwater tracer and in situ isotope enrichment study. Limnology and Oceanography: 46: 1977–1989.

Tobias, C.R., Cieri, M., Peterson, B.J., Deegan, L.A., Vallino, J., Hughes, J. 2003. Processing watershed-derived nitrogen in a well-flushed New England estuary. Limnology and Oceanography: 48(5), 1766–1778.

Tollner, E.W., Kazanci, C.K. 2007. Defining an ecological thermodynamics using discrete simulation approaches. Ecological Modelling: 208, 68-79.

Tourna, M., Freitag, T.E., Nicol G.W., Prosser, J.I. 2008. Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. Environmental Microbiology: 10(5), 1357-1364.

Tzoraki O., Nikolaidis, N.P., Amaxidis, Y., Skoulikidis, N.T. 2007. In stream biogeochemical processes of a temporary river. Environmental Science and Technology: 41, 1225–1231.

Unkovich, M., Pate, J., McNeill, A., Gibbs, D.J. (Eds.). 2001. Stable Isotope Techniques in the Study of Biological Processes and Functioning of Ecosystems. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Vadeboncoeur, Y. and Vander Zanden, M.J. 2002. Putting the Lake Back Together: Reintegrating Benthic Pathways into Lake Food Web Models. BioScience: 52 (1), 44-54.

Vadeboncoeur, Y., Jeppesen, E., Vander Zanden, M.J., Schierup, H., Christoffersen, K., Lodge, D.M. 2003. From Greenland to green lakes: cultural eutrophication and the loss of benthic energy pathways in lakes. Limnology and Oceanography: 48,1408–1418.

Vander Zanden, M.J., Shuter, B.J., Lester, N., Rasmussen, J.B.1999. Patterns of food chain length in lakes: a stable isotope study. The American Naturalist: 154, 406–416.

Vander Zanden, M.J. and Rasmussen, J.B. 2001. Variation in $\delta^{15}N$ and $\delta^{13}C$ trophic fractionation: implications for aquatic food web studies. Limnology and Oceanography: 46, 2061–2066.

Vander Zanden, M.J. and Vadeboncoeur, I. 2002. Fishes as integrators of benthic and pelagic food webs in lakes. Ecology: 83, 2152–2161.

Vander Zanden, M.J., Chandra, S., Allen, B.C., Reuter, J.E., Goldman, C.R. 2003. Historical Food Web Structure and Restoration of Native Aquatic Communities in the Lake Tahoe (California–Nevada) Basin. Ecosystems: 6(3), 274-288.

Vander Zanden, M.J. and Fetzer, W.W. 2007. Global patterns of aquatic food chain length. Oikos: 116, 1378-1388.

Van den Meersche, K., Soetaert, K., Middelburg, J.J. 2011. Plankton dynamics in an estuarine plume: a mesocosm ^{13}C and ^{15}N tracer study. Marine Ecology Progress Series: 429, 29–43.

Van Oevelen, D.V., Middleburg, J.J., Soetaert, K., Moodley, L. 2006a. The fate of bacterial carbon in an intertidal sediment: modelling an in situ isotope tracer experiment. Limnology and Oceanography: 51, 1302-1314.

Van Oevelen, D.V., Moodley, L., Soetaert, K., Middleburg, J.J. 2006b. The trophic significance of bacterial carbon in a marine intertidal sediment: results of an in situ stable isotope labelling study. Limnology and Oceanography: 5, 2349-2359.

Vanderklift, M. A. and Ponsard, S. 2003. Sources of variation in consumer-diet delta ^{15}N enrichment: A meta-analysis. Oecologia: 136, 169-182.

Vannote, R. L., Minshall, G.W., Cummings, K.W., Sedell, J.R., Cushing C.E. 1980. The River Continuum Concept. Canadian Journal of Fisheries and Aquatic Sciences: 37, 130-137.

Vitousek, P.M. and Farrington, H. 1997. Nutrient limitation and soil development: Experimental test of a biogeochemical theory. Biogeochemistry: 3(7), 63–75.

Velinsky. D, J., Burdige, D, 1., and Fogel. M. L. 1991. Nitrogen Diagenesis in Anoxic Marine Sediments: Isotope Effects. Annual Report to the Director. Geophysics Laboratory, Carnegie Institution, Washington, DC, 1990-1991.

Velinsky, D. J., and Fogel, M.L. 1999. Cycling of dissolved and particulate nitrogen and carbon in the Framvaren Fjord, Norway: Stable isotopic variations. Marine Chemistry: 67, 161–180.

Ventura, M. and Jeppesen, E. 2009. Effects of fixation on freshwater invertebrate carbon and nitrogen isotope composition and its arithmetic correction. Hydrobiologia: 632, 297–308.

Veuger, B., Van Oevelen, D.V., Boschker, H.T., Middleburg, J.J. 2006. Fate of peptidoglycan in an intertidal sediment: an in situ ^{13}C-labeling. Limnology and Oceanography: 51, 1572-1580.

Veuger, B., Eyre, B.D., Maher, D., Middleburg, J.J. 2007. Nitrogen incorporation and retention by bacteria, algae, and fauna in a subtropical

intertidal sediment: an in situ ^{15}N-labeling study. Limnology and Oceanography: 52, 1930–1942.

Vila-Costa, M., Bartrons, M., Catalan, J., Casamayor, E.O. 2014. Nitrogen-Cycling Genes in Epilithic Biofilms of Oligotrophic High-Altitude Lakes (Central Pyrenees, Spain). Microbiology Ecology: 68, 60–69.

Vilar-Sanz, A., Puig, S., García-Lledó, A., Trias, R., Balaguer, M.D., Colprim, J., Bañeras, L. 2013. Denitrifying Bacterial Communities Affect Current Production and Nitrous Oxide Accumulation in a Microbial Fuel Cell. PLoS ONE: 8(5), e63460.

Vitousek, P.M., Cassman, K., Cleveland, C., Crews, T., Field, C.B., Grimm, N.B., Howarth, R.W., Marino, R., Martinelli, L., Rastetter, E.B., and others. 2002. Towards an ecological understanding of biological nitrogen fixation. Biogeochemistry: 57, 1–45.

Vitousek, P.M., Aber, J.D., Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger W.H., Tilman, D.G. 1997. Human alteration of the global nitrogen cycle: sources and consequences. Ecological Applications: 7, 737–750.

Wagner, M. and Loy, A. 2002. Bacterial community composition and function in sewage treatment systems. Current Opinion in Biotechnology: 13, 218–227.

Ward, B.B. 1996. Nitrification and Denitrification: Probing the Nitrogen Cycle in Aquatic Environments. Microbiology Ecology: 32, 247-261.

Ward, B.B., Devol, A.H., Rich, J.J., Chang, B.X., Bulow, S.E., Naik, H., Pratihary, A., Jayakumar, A. 2009. Denitrification as the dominant nitrogen loss process in the Arabian Sea. Nature: 461(8276), 78-82.

Warren, P.H. 1990. Variation in food-web structure: the determinants of connectance. The American Naturalist: 136, 689–700.

Warren, P.H. 1994. Making connections in food webs. Trends in Ecology and Evolution: 9, 136-141.

Webster, J.R. 1975. Analysis of potassium and calcium dynamics in stream ecosystems in three southern Appalachian watersheds of contrasting vegetation. Dissertation. University of Georgia, Athens, Geoirgia. USA.

Webster, J.R., and Pattern, B.C. 1979. Effects of watershed perturbation on stream potassium and calcium dynamics. Ecological Monographs: 19, 51-71.

Webster, J. R. and Ehrman, T. P. 1996. Solute dynamics. In Methods in Stream Ecology. Hauer, F. R. and Lamberti, G. A. (eds). Academic Press, New York

Weidel, B.C., Carpenter, S.R., Cole, J.J., Hodgson, J.R., Kitchell, J.F., Pace, M.L., Solomon, C.T. 2008. Carbon sources supporting fish growth in a north temperate lake. Aquatic Science: 70, 446–458.

Weidel, B.C., Carpenter, S.R., Kitchell, J.F., Vander Zanden, M.J. 2011. Rates and components of carbon turnover in fish muscle: insights from bioenergetics models and a whole-lake ^{13}C addition. Canadian Journal of Fisheries and Aquatic Science: 68, 387–399.

Wetzel, R.G and Likens, G. E. 2000. Limnological Analyses. Springer, Nueva York

Wetzel, R.G. 2001. Limnology: Lake and River Ecosystems. Academic, San Diego, Calif.

Wilcox, H.S., Wallace, J.B., Meyer, J.L., Benstead, J.P. 2005. Effects of labile carbon addition on a headwater stream food web. Limnology and Oceanography: 50, 1300-1312.

Winning, M.A., Connolly, R.M., Loneragan, N.R., Bunn, S.E. 1999. ^{15}N enrichment as a method of separating the isotopic signatures of seagrass and its epiphytes for food web analysis. Marine Ecology Progress Series 189, 289–294

Winemiller, K. O. 1990. Spatial and temporal variation in tropical fish trophic networks. Ecological Monographs: 60, 331–36.

Winemiller, K.O. and C.A. Layman. 2005. Food web science: moving on the path from abstraction to prediction. In Dynamic Food Webs: Multispecies Assemblages, Ecosystem Development and Environmental Change, P.C. de Ruiter, V. Wolters, and J.C. Moore, (eds). Elsevier, Amsterdam.

Wollheim, W.M., Peterson, B.J., Deegan, L.A., Bahr, M., Hobbie, J.E., Jones, D., Bowden, W.B., Hersey, A.E., Kling, G.W., Miller, M.C. 1999. A couple field and modeling approach for the analysis of nitrogen cycling streams. Journal of the North American Benthological Society: 18, 199-221.

Wollheim, W.M., Peterson, B.J., Deegan, L.A., Hobbie, J.E., Hooker, B., Bowden, W.B., Edwardson, K.J., Arscott, D.B., Hershey, A.E., Finlay, J.

2001. Influence of stream size on ammonium and suspended particulate nitrogen processing. Limnology and Oceanography: 46,1-13.

Worm, B. and Duffy, J.E. 2003. Biodiversity, productivity, and stability in real food webs. Trends in Ecology and Evolution: 18, 628–632.

Yodzis, P. 1980. The connectance of real ecosystems. Nature: 284, 544-545.

Yoshinaga, I., Amano, T., Yamagishi, T., Okada, K., Ueda, S., Sako, Y., Suwa, Y. 2011. Distribution and diversity of anaerobic ammonium oxidation (anammox) bacteria in the sediment of a eutrophic freshwater lake, Lake Kitaura, Japan. Microbes and Environments: 26 (3), 189–197.

Young, R.G., Huryn, A.D. 1998. Comment: improvements to the diurnal upstream-downstream dissolved oxygen change technique for determining whole-stream metabolism in small streams. Canadian Journal of Fisheries and Aquatic Science: 55,1784–1785.

Yoshii, K., Melnik, N.G., Timoshkin, O.A., Bandarenko, N.A., Anoshko, P.N., Yoshioka, T., Wada, E. 1999. Stable isotope analyses of the pelagic food web in Lake Baikal. Limnology and Oceanography: 44, 502-511.

Yu, J., Li, Y., Liu, X., Li, K., Chen, F., Gulati, R., Liu, Z. 2013. The fate of cyanobacterial detritus in the food web of Lake Taihu: a mesocosm study using ^{13}C and ^{15}N labeling. Hydrobiologia: 710, 39-46.

Zar, J.H. 1996. Biostatistical analysis. Prentice Hall, Upper Saddle River.

Zarnetske, J.P., Haggerty, R., Wondzell, S.M., Baker, M.A. 2011. Labile dissolved organic carbon supply limits hyporheic denitrification. Journal of Geophysical Research:116, G04036.

Zhang, T. and Fang, H.H.P. 2005. 16S rDNA clone library screening of environmental samples using melting curve analysis. Journal of the Chinese Institute of Chemical Engineers: 28, 1153–1155.

Zhang, L., Li, K.W., Liu, Z.Y., Middelburg, J.J. 2010. Sedimented cyanobacterial detritus as a source of nutrient for submerged macrophytes (*Vallisneria spiralis* and *Elodea nuttallii*): an isotope labeling experiment using ^{15}N. Limnology and Oceanography: 55,1912-1917.

Zumft, W.G. 1997. Cell biology and molecular basis of denitrification. Microbiology and Molecular Biology Reviews: 61, 533–616.

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